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PURINERGIC SIGNALING IN ACTIVATED VENTRICULAR MYOFIBROBLASTS AS A PUTATIVE PHARMACOLOGICAL TARGET TO PREVENT CARDIAC REMODELING

Tese de Candidatura ao grau de Doutor em Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Dissertation in fulfilment of the requirements for the PhD degree in Biomedical Sciences, submitted to Instituto de Ciências Biomédicas Abel Salazar of University of Porto.

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ABBREVIATIONS

$[Ca^{2+}]_i$, intracellular calcium
2-APB, 2-aminoethoxydiphenylborane
ABC, ATP-binding cassette
AC, adenylyl cyclase
ADP, adenosine 5′-diphosphate
ADPRC, ADP-ribosylcyclase
ADPßS, adenosine 5'-(α,β-methylene) diphosphate
AMP, adenosine 5'-monophosphate
AngII, angiotensin II
AR-C 66096, 2-(propylthio)adenosine-5'-O-(ß,γ-difluoro methylene) triphosphate tetrasodium salt
ATP, adenosine 5′-triphosphate
ATPγS, adenosine 5′-[γ-thio]triphosphate
BCA, bicinchoninic acid
BFA, brefeldin A
BSA, bovine serum albumin
Ca^{2+}, calcium ion
cADPR, cyclic adenosine diphosphate ribose
CBX, carbenoxolone
CF, cardiac fibroblasts
Chelerytrine, 1,2-Dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride
Cx43, connexin 43
DAG, diacylglycerol
DDR2, discoidin domain receptor 2
DMEM, dulbecco's modified eagle medium
DMSO, dimethylsulphoxide;
E-NPPs, ectonucleotide pyrophosphatase and/or phosphodiesterases
E-NTPDases, ectonucleoside triphosphate diphosphohydrolases
ECM, extracellular matrix
EDTA, ethylenediaminetetraacetic acid

EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N acidtetraacetic acid

EMT, epithelial-mesenchymal-transition

EndMT, endothelialmesenchymal-transition

EPAC, exchange protein directly activated by cAMP

ESI-09, α-[2-(3-chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-b-oxo-3-isoxazolepropanenitrile

ET-1, endothelin-1

FBS, fetal bovine serum

FGF-2, fibroblasts growth factor 2

FSP1, fibroblasts specific protein 1

GPCR, G-protein-coupled receptor

H-89, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride

H1152, (S)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride

IBMX, 1-methyl-3-isobutylxanthine

IGF1, insulin-like growth factor 1

IP_3, inositol 1,4,5-triphosphate

KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate

MAPK, mitogen-activated protein kinase

MDL12,330a, cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride

MFQ, Mefloquine

MI, myocardial infarction

MRS 2179, 2'-deoxyN6-methyladenosine 3',5'-biphosphate tetrasodium salt

MRS 2211, 2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridine carboxaldehyde disodium salt

MRS 2365, [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt

MRS 2578, N,N''-1,4-butanediylbis[N'-(3-isothiocyanatophenyl)thiourea

MRS 2768, uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt

MRS 4062, N^4-phenylpropoxycytidine-5'-O-triphosphate tetra(triethylammonium) salt

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

MβCD, methil-ß-cyclodextrin

NAD, nicotinamide adenine nucleotide

NF 340, 4,4'-(Carbonylbis(imino-3,1-(4-methyl-phenylene)carbonylimino))bis (naphthalene-2,6-disulfonic acid) tetrasodium salt

NF 546, 4,4'-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene) carbonylimino))-bis-1,3-xylene-alpha, alpha′-diphosphonic acid tetrasodium salt

NFAT, nuclear factor of activated T cells

ORM-10103, 2-[(3,4-dihydro-2-phenyl-2H-1-benzopyran-6-yl)oxy]-5-nitro-pyridine

Panx, pannexin 1

PBS, phosphate buffered saline system

PDGF-α, platelet-derived growth factor-α

PFA, paraformaldehyde

PIP_2, PtIns(4,5)P2

PKA, protein kinase A

PKC, phosphokinase C

PLC, phospholipase C

RB-2, reactive blue-2

RT-PCR, reverse transcription-polymerase chain reaction

SQ 22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine

TCF21, transcription factor 21

TGFβ, transforming growth factor β

TRP, transient potential receptors

U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione

UDP, uridine 5'-diphosphate

UTP, uridine 5'-triphosphate

α-SMA, α-smooth muscle actin

RESUMO

Os fibroblastos cardíacos desempenham um papel crucial na remodelação do coração assim como nas alterações electrofisiológicas e hemodinâmicas após enfarte do miocárdio. Contudo, a sua participação na homeostasia cardíaca na saúde e na doença tem sido preterida em favor do papel central dos cardiomiócitos enquanto responsáveis pela contratilidade e sinalização elétrica do miocárdio. Durante a isquemia e reperfusão do miocárdio, são libertadas grandes quantidades de ATP e UTP para o meio extracelular, ativando múltiplos subtipos de recetores purinérgicos P2. Uma vez no meio extracelular, estes nucleótidos podem ser rapidamente desfosforilados em nucleósidos difosfato, por NTPDases ligadas à membrana plasmática, permitindo a ativação subsequente de recetores P2Y sensíveis ao ADP ou UDP. Os fibroblastos cardíacos expressam ainda uma variedade de transportadores e canais permeáveis ao cálcio, tais como canais TRP e o trocador Na^+/Ca^{2+}, que podem regular os efeitos produzidos na sinalização mediada pelo Ca^{2+} operada por recetores purinérgicos. Este sistema dinâmico que abrange os locais de libertação dos nucleótidos, enzimas membranares, recetores purinérgicos e canais iónicos deve funcionar de forma muito coordenada para regular várias das funções celulares. Neste contexto, o presente estudo contribuiu para explorar o papel da sinalização purinérgica sobre os aumentos transitórios de Ca^{2+} e sobre a proliferação / diferenciação de fibroblastos ventriculares isolados do coração de ratazanas adultas.

Demonstrámos que os fibroblastos ventriculares em cultura co-expressam o recetor do domínio discoide 2 e actina-α do músculo liso e, por isso, possuem um fenótipo ativado semelhante aos miofibroblastos encontrados no coração após fenómenos de isquemia/reperfusão, inflamação e sobrecarga de pressão ou volume. Tanto os nucleótidos de adenina como os de uracilo causaram a elevação dos níveis intracelulares de cálcio ($[Ca^{2+}]_i$) de forma bifásica. *i.e.* observou-se numa primeira fase um aumento rápido dos níveis de $[Ca^{2+}]_i$ através do seu recrutamento a partir de reservatórios intracelulares sensíveis à tapsigargina, cujos níveis diminuiram parcialmente para um patamar que foi mantido através da entrada de Ca^{2+} do meio extracelular. O UTP e o ATP foram

os nucleótidos mais potentes, seguidos do ADP e UDP por essa ordem. O recetor responsável pela resposta rápida de $[Ca^{2+}]_i$ nos fibroblastos cardíacos é provavelmente do subtipo $P2Y_4$. Esta conclusão foi retirada (1) pela presença deste recetor nestas células verificada por microscopia confocal, (2) pela reprodução do efeito do UTP pelo agonista seletivo dos recetores $P2Y_4$, MRS4062, e (3) pelo antagonismo da resposta aos nucleótidos tanto pelo reactive blue-2 (RB-2) como pela suramina. A demonstração do envolvimento da via de transdução de sinal intracelular associada à fosfolipase C com libertação de IP_3 e DAG a partir dos fosfolípidos de membrana reforça a ideia do envolvimento de um recetor acoplado à proteína $G_{q/11}$, tal como o recetor $P2Y_4$

Os resultados deste estudo mostram pela primeira vez que a ativação de recetores $P2Y_4$ pelo UTP facilita a libertação de ATP para o meio extracelular através de hemicanais contendo Cx43. Este processo promove a ativação de recetores $P2Y_{11}$ sensíveis ao ATP. O sinergismo entre a ativação de recetores $P2Y_4$ e $P2Y_{11}$ acoplados a proteínas $G_{q/11}$ e G_s, respetivamente, favorece a manutenção dos níveis de $[Ca^{2+}]_i$ na segunda fase da resposta ao UTP. Esta conclusão foi retirada a partir do bloqueio da fase de planalto da resposta ao UTP (1) na presença do antagonista seletivo dos recetores $P2Y_{11}$, NF340, e (2) após o tratamento das células com inibidores da ciclase do adenilato, SQ22536 ou MDL12,3303a.

Apesar de se ter detetado a presença de recetores sensíveis ao ADP, $P2Y_1$, $P2Y_{12}$ e $P2Y_{13}$, nos fibroblastos ventriculares, o efeito deste nucleótido sobre a acumulação de $[Ca^{2+}]_i$ nestas células possui uma farmacologia compatível com a ativação preferencial de recetores do subtipo $P2Y_4$ mas, neste caso, sem um envolvimento significativo do recetor $P2Y_{11}$ na fase de planalto da resposta. Mostra-se pela primeira vez neste trabalho que a entrada capacitativa de Ca^{2+} responsável pela segunda fase da resposta ao ADP depende da abertura de canais TRP (sensíveis ao 2-APB e ao ácido flufenâmico) aglomerados junto aos recetores $P2Y_4$ e à fosfolipase C em microdomínios membranares ricos em colesterol e caveolina-1 – jangadas lipídicas (*lipid rafts*). A disrupção das jangadas lipídicas motivada pela depleção de colesterol com metil-β-ciclodextrina eliminou a segunda fase do sinal de $[Ca^{2+}]_i$ causado pelo ADP

O efeito a longo prazo dos nucleótidos de adenina e uracilo sobre a proliferação e diferenciação dos fibroblastos ventriculares em cultura mostrou o envolvimento preferencial de recetores dos subtipos $P2Y_2$ e $P2Y_1$, consoante a ativação se produza por nucleósidos trifosfatados (ATP e UTP) e difosfatados (ADP), ou pelos seus análogos estáveis MRS2768 e ADPβS, respetivamente. Qualquer um destes recetores possui um potencial efeito pró-fibrótico na remodelação miocárdica, sendo de destacar que a ativação tónica do recetor $P2Y_1$, mas não de qualquer outro recetor sensível ao ADP (e.g. $P2Y_{12}$ ou $P2Y_{13}$) promove a síntese de colagénio de tipo I em maior escala do que a ativação do recetor $P2Y_2$. Por se tratar de um recetor facilmente dessensibilizável através da ação repetida ou prolongada dos seus agonistas, o recetor $P2Y_4$ não exerce qualquer efeito significativo na proliferação dos fibroblastos cardíacos em cultura. O efeito do UTP sobre a proliferação de fibroblastos cardíacos em cultura parece ser em parte contrabalançado pela ativação tónica do recetor $P2Y_{11}$, já que o mesmo foi prevenido pelo antagonista seletivo destes recetores, NF340. O efeito anti-fibrótico do recetor $P2Y_{11}$ foi confirmado porque o seu agonista seletivo, NF546, reduziu a proliferação dos fibroblastos por um mecanismo dependente da via de transdução de sinal envolvendo a ciclase do adenilato / AMP cíclico / EPAC.

Em conclusão, os fibroblastos ventriculares de ratazanas adultas expressam diversos subtipos de recetores purinérgicos funcionais, tais como $P2Y_1$, $P2Y_2$, $P2Y_4$ e $P2Y_{11}$. Os nucleótidos de adenina e uracilo provocam oscilações de $[Ca^{2+}]_i$ através da ativação preferencial dos recetores $P2Y_4$. Para além do recrutamento rápido de Ca^{2+} a partir de reservatórios intracelulares, os níveis intracelulares de Ca^{2+} são mantidos em níveis elevados através da entrada Ca^{2+} do meio extracelular por intermédio de canais TRP localizados junto aos recetores em microdomínios (*lipid rafts*) da membrana plasmática. A ativação seletiva do recetor $P2Y_4$ conduz, ainda, à libertação de ATP e, subsequente, ativação de recetores $P2Y_{11}$ que também contribui para a segunda fase da sinalização mediada pelo Ca^{2+}. Os nucleótidos de adenina e uracilo possuem uma atividade pró-fibrótica favorecida pela ativação de recetores $P2Y_1$ e $P2Y_2$. Este efeito promotor da proliferação fibroblástica pode ser contrariado pela ativação de recetores do subtipo $P2Y_{11}$, cujo potencial anti-fibrótico pode ser aproveitado clinicamente usando novos ativadores específicos.

ABSTRACT

Cardiac fibroblasts play crucial roles in heart remodeling, electrophysiology and hemodynamic alterations following myocardial infarction. Notwithstanding this, research has been focused predominantly on the pathophysiological role of cardiomyocytes, which can be explained because these cells were considered, until recently, the main determinants of contractility and electrical integration of the myocardium. During myocardial ischemia and reperfusion, both ATP and UTP are released in huge amounts into the extracellular milieu, leading to the activation of many subtypes of P2 purinoceptors. Once in the extracellular milieu, these nucleotides can be rapidly dephosphorylated into diphosphate nucleosides by membrane-bound NTPDases, which facilitate the activation of ADP- or UDP-sensitive P2Y receptors. Besides that, cardiac fibroblasts are also endowed with a variety of calcium channels and transporters, like transient receptor channels (TRP) and the Na^+/Ca^{2+} exchanger, which may modulate Ca^{2+} signals operated by P2 purinoceptors activation. This dynamic system covering nucleotide release sites, membrane-bound enzymes, purinoceptors and ion channels must function coordinately in order to regulate various cell functions. In this context, this study was designed to explore the role of purinergic signaling on Ca^{2+} transients and proliferation / differentiation of fibroblasts isolated from adult rat ventricles.

Cultured ventricular fibroblasts co-express discoidin domain receptor 2 and alpha-smooth muscle actin, suggesting a myofibroblast (activated) phenotype. Both adenine and uracil nucleotides caused a biphasic intracellular calcium ($[Ca^{2+}]_i$) response consisting of a fast $[Ca^{2+}]_i$ rise originated from thapsigargin-sensitive internal stores, which partially declined to a plateau providing that Ca^{2+} was present in the extracellular fluid. $[Ca^{2+}]_i$ responses caused by UTP and ATP were more exuberant than those produced by ADP and UDP, in that order. The receptor responsible for the fast $[Ca^{2+}]_i$ response in ventricular myofibroblasts is of the $P2Y_4$ subtype. This conclusion was drawn from the presence of this receptor demonstrated by confocal microscopy, and because the effect of UTP was reproduced by the $P2Y_4$ selective agonist, MRS4062, and antagonized by both reactive blue-2 (RB-2) and suramin. The $P2Y_4$ receptor is usually coupled to $G_{q/11}$ protein and to phospholipase C resulting in the release of IP_3 and DAG from

membrane phospholipids, and this mechanism was proved to be involved in ventricular fibroblasts in culture.

Results from this study show for the first time that activation of the $P2Y_4$ receptor by UTP facilitates the release of ATP to the extracellular milieu through hemichannels containing Cx43. Extracellular ATP accumulation favors activation of the $P2Y_{11}$ receptor that is co-expressed with the $P2Y_4$ in ventricular myofibroblasts. Synergism between $P2Y_4$ and $P2Y_{11}$ receptors coupling to $G_{q/11}$ and G_s proteins, respectively, contributes to maintain high $[Ca^{2+}]_i$ levels during the plateau phase of the Ca^{2+} response to UTP. This was concluded because blockage of UTP-induced $[Ca^{2+}]_i$ plateau was verified (1) in the presence of a selective $P2Y_{11}$ receptor antagonist, NF340, and (2) after treating the cells with adenylate cyclase inhibitors, such as SQ22536 or MDL12,3303a.

Besides the fact that ventricular fibroblasts are endowed with ADP-sensitive receptors, namely $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$, the pharmacology of ADP-mediated effects in these cells is compatible with a preferential activation of the $P2Y_4$ receptor subtype, without a significant involvement of the $P2Y_{11}$ receptor in the plateau phase of the $[Ca^{2+}]_i$ response. We show here for the first time that the capacitative Ca^{2+} entry responsible for the second phase of the ADP response depends on 2-APB- and flufenamic acid-sensitive TRP channels opening. These channels are compartmentalized with the $P2Y_4$ receptor and phospholipase C within lipid rafts microdomains enriched in cholesterol and caveolin-1. Disruption of lipid rafts microdomains by depletion of cholesterol with methyl-β-cyclodextrin abrogated the second plateau phase of ADP-induced $[Ca^{2+}]_i$ response.

Long-term effects of adenine and uracil nucleotides on proliferation and/or differentiation of cultured ventricular fibroblasts are mediated preferentially via $P2Y_2$ and $P2Y_1$ receptor subtypes depending whether native ligands are nucleoside triphosphates (both ATP and UTP) or diphosphates (ADP), or their stable analogs MRS2768 and ADPβS, respectively. Both receptors have a pro-fibrotic potential on cardiac remodeling, but it is worth to note that tonic activation of the $P2Y_1$ receptor, but no any other receptor sensitive to ADP activation (e.g. $P2Y_{12}$ or $P2Y_{13}$) favors type I collagen synthesis more effectively than the $P2Y_2$ receptor subtype. Given that the $P2Y_4$ receptor is fast desensitizing by repeated or prolonged application of its agonists, this receptor subtype had no effect on cardiac fibroblast cells growth in culture. Interestingly, the proliferative effect of

UTP may be partially counteracted by tonic activation of the $P2Y_{11}$ receptor, which can be blocked selectively by NF340 or by inhibiting the adenylate cyclase / cyclic AMP / EPAC transduction pathway.

In conclusion, ventricular fibroblasts from adult rats are endowed with several P2Y receptor subtypes, namely $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$. Adenine and uracil nucleotides cause $[Ca^{2+}]_i$ transients through the preferential activation of $P2Y_4$ receptors. Besides the fast $[Ca^{2+}]_i$ rise originated by Ca^{2+} recruitment from internal stores, capacitative Ca^{2+} entry from the extracellular milieu sustains elevated $[Ca^{2+}]_i$ levels through opening of TRP channels compartmentalized with the $P2Y_4$ receptor within lipid rafts membrane microdomains. In addition, selective activation of the $P2Y_4$ facilitates the outflow of ATP, which accumulation in the extracellular milieu favors activation of $P2Y_{11}$ receptors also contributing to the second (plateau) phase of UTP-induced $[Ca^{2+}]_i$ response. Adenine and uracil nucleotides display an overall pro-fibrotic activity that is predominantly mediated by $P2Y_1$ and $P2Y_2$ receptors activation. Fibroblast growth promotion can be, at least partially, counteracted by $P2Y_{11}$ activation and we propose here that the anti-fibrotic effect of $P2Y_{11}$ receptors might be used as a novel therapeutic target to prevent cardiac fibrosis.

CHAPTER 1. INTRODUCTION

1.1. Cardiac remodeling

1.1.1. Cardiac remodeling: an introduction

Heart diseases are a major cause of morbidity and mortality in economically developed countries. These disease conditions are associated with pathological remodeling of the heart, an event that is characterized by changes in gene expression leading to structural alterations at molecular, cellular and interstitial levels that are manifested clinically as changes in size, shape and activity of the heart (Cohn et al., 2000). These remodeling events may be secondary to myocardial infarction (MI), pressure/volume overload and inflammatory insults, or may be idiopathic as observed in dilated cardiomyopathy. Although there are several different heart diseases with various pathophysiological mechanisms, they share common molecular, biochemical, and cellular mechanisms to collectively change the shape and function of the myocardium (Kehat et al., 2010). In what concerns the clinical diagnosis, usually heart remodeling is based on the detection of morphological changes, such as modifications in the diameter of the cavities, cardiac mass (hypertrophy and atrophy), geometry (heart wall thickness and shape), areas of scar after MI, fibrosis and inflammatory infiltrates (Cohn et al., 2000). Collectively, these features result in heart dysfunction leading to a poor prognosis.

The remodeling process of the heart may be divided into different phases. Despite the initial remodeling phase leading to reparation of the necrotic area and to scar formation may be, in some extension, considered beneficial (Cohn et al., 2000), when faced with increased workload or injury, the cardiac remodeling begins to become pathological and cardiac functions decline often leading to heart failure (Cohn et al., 2000; Kehat et al., 2010).

The macroscopic and functional modifications of the heart described above result from processes that involve cellular and molecular cardiac changes, such as cardiomyocyte hypertrophy, necrosis (Tan et al., 1991), apoptosis (Sharov et al., 1996; Teiger et al., 1996; Olivetti et al., 1997), fibrosis (Anderson et al., 1979), increased fibrillary collagen (Weber et al., 1990) and fibroblast transdifferentiation and proliferation (Villareal et al., 1993). In general, the critical phases of this

response consist in inflammation, proliferation of non-myocyte cells, and scarring (Travers et al., 2016).

In what concerns the mechanisms underlying cardiac remodeling, as well as cardiac function in healthy tissues, most of the studies rely on cardiomyocyte function, whereas stromal cells, such as cardiac fibroblasts, tend not to be considered. In fact, this is not surprising given that the heart pumping function directly depends on the contractile activity of cardiomyocytes. However, to effectively perform their functions, cardiomyocytes have to be embedded in an appropriately configured extracellular matrix (ECM) and to coordinate their activity with non-myocyte cardiac cells, mainly cardiac fibroblasts.

1.1.2. Cardiac remodeling: fibrosis

As mentioned earlier, myocardial fibrosis constitutes one of the cardiac morphological changes detected during heart remodeling. In fact, myocardial fibrosis is one of the most common histologic features of the failing heart and constitutes a terminal event of various cardiac diseases. An important consideration is that the term ‘fibrosis’ is often confused with ‘scar’ and these terms are frequently used interchangeably. However, we must consider that fibrosis is not a synonymous of an increased presence of interstitial cells but, conversely, it is a measurable characteristic quantified through the presence of collagen - a key component of the acellular fraction of connective tissue. Thus, fibrotic scars, for instance commonly observed in the skin, are generally acellular and predominantly composed of fibrillar collagen (Desmoulière et al., 1995). In the heart, however, scar tissue assumes a more proactive role than simply preserving ventricular integrity, facilitating force transmission, and preventing wall rupture. This ability led some authors to designate cardiac scars as ‘very much alive’ scars because of the heterocellular activity that contributes to the maintenance of structural and mechanical integrity following heart injury (Rog-Zielinska et al., 2016).

Adaptive cardiac fibrosis is a cellular and molecular process that aims at maintaining the structural integrity and pressure-generating capacity of the heart. This process can be categorized into 2 classical forms, namely reactive interstitial fibrosis or replacement fibrosis, according to the underlying cardiomyopathic process. The term ”reactive interstitial fibrosis” is used to describe expansion of

the cardiac interstitial space in the absence of significant loss of cardiomyocytes. This type of fibrosis presents a diffuse distribution within the interstitium that is more often perivascular (Weber et al., 1988). In addition, this process has a progressive onset and follows the increase in collagen synthesis by myofibroblasts under the influence of several stimuli. Thus, interstitial fibrosis is an intermediate marker of disease severity, as it has been shown in hypertensive cardiomyopathy, and it precedes irreversible replacement fibrosis (Webber et al., 1991; Martos et al., 2007). In contrast, ”reparative or replacement fibrosis” refers to the substitution of cardiomyocytes after cell damage or necrosis by plexiform fibrosis, mainly type I collagen (Sutton et al., 2000). Replacement fibrosis appears as soon as the cardiomyocytes integrity is affected and it may have a localized or a diffuse distribution according to the etiology (Mahrholdt et al., 2005; Bohl et al., 2008; Karamitsos et al., 2009). In addition, it has been also described a third subtype of fibrosis designated ”infiltrative interstitial fibrosis” that is induced by the progressive deposition of insoluble proteins (amyloidosis) or glycosphingolipids (Anderson Fabry’s disease) (Shah et al., 2006; Zarate et al., 2008) in the cardiac interstitium.

As mentioned above, despite different pathophysiological events can lead to a common process designated cardiac remodeling, it can assume distinctive forms. For example, in the early stages of infarct healing, fibroblasts become pro-inflammatory cells, producing cytokines, chemokines and proteases that delay myofibroblast transformation until the wound is cleared from dead cells and matrix debris. After the initial inflammatory phase of healing, fibroblasts undergo phenotypic changes mounting the proliferative phase of healing. This stage is characterized by the removal of pro-inflammatory signals, high proliferative activity, migration through the provisional matrix network of the infarct, expression and incorporation of contractile proteins in the cytoskeleton and synthesis of both structural and matricellular ECM proteins (Shinde and Frangogiannis, 2014). Formation of a collagen-based matrix marks the end of the proliferative phase and sets the period for maturation, a poorly understood process that is characterized by matrix cross-linking and progressive loss of the cellular elements. Conversely, the non-infarcted myocardium is subjected to pressure and volume overload causing chronic activation of the local fibroblast population. Pressure overload

induces early activation of matrix synthetic pathways, associated with fibrosis and diastolic dysfunction, followed by activation of matrix-degrading signals, chamber dilatation and predominantly associated with matrix loss and cardiac dilatation.

In advanced phases of the fibrotic process, the tensile strength of collagen increases within the site of injury (van den Borne et al., 2010). In this setting, a subset of activated myofibroblasts acquire new phenotypic characteristics, including the expression of the contractile protein α-smooth muscle actin (α-SMA), and contribute to pathological cardiac remodeling (Tomasek et al., 2002; Calderone et al., 2006). Although initially adaptive, these processes eventually lead to the development of adverse changes in ventricular structure and compliance, and a concurrent progression into overt heart failure.

1.1.3. Cardiac remodeling: the role of cardiac fibroblasts

Despite the pathophysiological importance of fibrosis in cardiovascular disease, as well as the predominant functions of cardiac fibroblasts in the process, these cells remain relatively mischaracterized and poorly understood. In fact, cardiac fibroblasts have preponderant functions on the cardiac remodeling process, particularly fibrosis, but also in the regulation of healthy cardiac physiology. For instance, after an acute myocardial injury, the expression of various proinflammatory cytokines and profibrotic factors is up-regulated in cardiac fibroblasts, leading to increased proliferation of these cells and, ultimately, the transition to the myofibroblast phenotype (Kawaguchi et al., 2011). During this maturation phase, myofibroblasts, sometimes referred as hipersecretory cells, begin to secrete increased levels of collagen (particularly, type I and III collagen) and other ECM proteins, including fibronectin and laminin. This activated phenotype of cardiac fibroblasts, which is not normally present in the adult heart, is thought to derive primary from resident interstitial and adventitial fibroblasts undergoing a phenotype switch, from bone marrow-derived circulating precursors (fibrocytes), and from cells undergoing epithelial/endothelial-to-mesenchymal transition (Hinz et al., 2007; Turner and Porter, 2013).

Pathological cardiac remodeling is characterized by fibroblasts accumulation and excessive deposition of ECM proteins, which leads to distorted organ architecture and has significant consequences on cardiac function (Weber, 2000). In addition, excess ECM and fibroblasts impair mechano-electric coupling

of cardiomyocytes, thus reducing cardiac contraction and increasing the risk of arrhythmogenesis and mortality (de Bakker et al., 1996; Spach and Boineau, 1997). In fact, the infiltration of myocardium with abundant myofibroblasts may alter cardiac electrophysiology by creating a barrier that blocks propagation of the electrical impulse, delaying conduction and promoting re-entry circuits. Thus, the dynamic phenotypic alterations of the fibroblast population following an infarction may profoundly influence arrhythmogenesis through several distinct mechanisms: secretion of pro-inflammatory mediators that may exert paracrine effects on cardiomyocytes, modifications of the expression of connexion-containing gap junctions, and incorporation of α-SMA into the fibroblast cytoskeleton thus contributing to arrhythmogenicity. Although fibrosis is strongly associated with an elevated risk of arrhythmogenesis, it is not well understood how exactly it is involved in either the active generation or the passive maintenance of abnormal electrical conduction episodes. Usually, the effect of connective tissue on cardiac electrophysiology is attributed to its non-excitability resulting in an electrical insulation of some areas of the heart. However, some clinical observations suggest that fibrotic tissue is not necessarily always and exclusively an electrical insulator. In fact, all aged hearts develop some degree of fibrosis (Biernacka and Frangogiannis, 2011), and one may wonder, therefore, why these do not appear to be arrhythmogenic suggesting the possibility of trans-scar conduction of electrical signals. These findings are in line with the concept of ‘very much alive’ scars of the heart, previously discussed. Furthermore, inflammation and fibrosis within perivascular regions may decrease tissue availability to oxygen and nutrients and, thus, increase the pathological remodeling response (Kai et al., 2006).

Another consideration is the importance of cell-cell communications as an integral feature of their behavior and function. In fact, cardiac fibroblasts do not operate in isolation but, on the other hand, these cells are particularly responsive to mechanical and chemical stimuli induced upon cardiac injury, which enhances the production and secretion of various pro-inflammatory and pro-fibrotic cytokines, growth factors, and chemokines (Kakkar and Lee, 2010). The released mediators act in both autocrine and paracrine fashions to modulate local inflammatory cells, cardiomyocytes, and cardiac fibroblasts themselves to exacerbate pathologic remodeling. In fact, many studies show that fibroblasts actively modulate the

structure and function of cardiomyocytes through different pathways: direct cell contact, via ECM proteins, and by the release of soluble mediators (Camelliti et al., 2005; Kakkar and Lee, 2010). On the other hand, cardiac fibroblasts activation and ECM proteins deposition may be affected by a number of humoral factors produced by other cell types including angiotensin II (AngII), endothelin-1 (ET-1), transforming growth factor β (TGF-β), fibroblasts growth factor 2 (FGF-2), and insulin-like growth factor 1 (IGF-1) (Bouzegrhane and Thibault, 2002; Manabe et al., 2002; Bujak and Frangogiannis, 2007).

1.2. Cardiac fibroblasts

1.2.1. Cardiac fibroblasts: origin, characteristics and function

Fibroblasts are mesenchymal cells, abundantly distributed in connective tissues of most organs that traditionally are viewed as matrix-producing cells which become activated following injury and participate in fibrotic tissue formation. Fibroblast cells are phenotypically diverse, and experimental evidence has demonstrated the existence of considerable heterogeneity between fibroblasts from different tissues (Camelliti et al., 2005). In what concerns cardiac fibroblast characteristic morphology identifiers, we may emphasize the lack of basement membrane and the presence of multiple elongated cytoplasmic processes or sheet-like extensions (Rog-Zielinska et al., 2016). However, their cellular characteristics also depend on their developmental stage and physiological conditions (Fries et al., 1994; Lekic et al., 1997). On the other hand, as fibroblasts are pleomorphic by nature, there is no defined threshold at which ‘a fibroblast becomes a myofibroblast’ because increased contractile filament content does not transform a fibroblast into a different cell type, and myofibroblasts do not have unique lineages separate from fibroblasts. In fact, the actin or myosin content and arrangement in cardiac fibroblasts can be affected by the environment, in particular mechanical parameters. Given that, some authors consider that the application of the term ‘myofibroblast’ may be more misleading than helpful (Camellitti et al., 2005).

Focus on cardiac fibroblast cells, they represent the most predominant cell type in cardiac tissue (about 70%) performing a variety of functions to maintain normal physiology (Porter and Turner, 2009). Cardiomyocytes, whilst fewer in

number, occupy the bulk volume of the myocardium and are contractile cells that provide mechanical force that is transmitted throughout ECM network. The most described function of cardiac fibroblasts is the maintenance of ECM homeostasis in the normal heart. To perform this function, the major constituents are the fibrillar collagens I (~80%) and III (~10%), with smaller amounts of collagens IV, V, VI, elastin, laminin, proteeoglycans, glycosaminoglycans, and others (Bosman and Stamenkovic, 2003). This process of regulation of ECM is achieved by the alternation of synthesis and degradation cycles.

One reason that leads to deficiency in the studies about cardiac fibroblasts is because of a lack of consistent cardiac fibroblast-cell markers. In fact, the absence of comprehensive markers has inhibited our ability to study the complex interactions between cardiac fibroblasts and the surrounding cells in vivo. An example of this is the intermediate filament protein, vimentin, which is often used as a fibroblasts marker, but it is expressed in many cell types including endothelial cells (Lane et al., 1983). Other molecules, such as CD90 (Thy1), Fibroblasts Specific Protein 1 (FSP1), platelet-derived growth factor-α (PDGF-α) and Periostin have been used as cardiac fibroblasts markers. However, several studies demonstrated that these molecules are frequently more associated to other cell types, such as immune cells, than fibroblasts (Kong et al., 2013; Moore-Morris et al., 2014). Transcription factor 21 (TCF21), a marker of pro-epicardium among other mesothelial populations, has also been used to identify fibroblasts in embryonic heart (Acharya et al., 2012). No one marker encompasses the combination of sensitivity and specificity to be definitive. In fact, there is not even a lineage marker currently in line that is specific to cardiac fibroblasts. Among arguably more robust markers is Discoidin Domain Receptors 2 (DDR2), also known as CD167b, which labels fibroblasts, but not endothelium, smooth muscle or myocytes (Goldsmith et al., 2004). The DDR2, as well as DDR1, represent a family of receptor tyrosine-specific kinase (RTK) that binds to collagen through its extracellular discoidin domain. These receptors have the ability to control a wide range of cellular responses including cell growth, differentiation, migration, metabolism and survival (Vogel et al., 1997). In what concerns the identification of activated fibroblasts (myofibroblasts), α-SMA is still the primary marker despite it is also expressed in vascular smooth muscle cells and pericytes, which are both

present in close proximity to fibroblasts, blighting its ability to distinguish fibroblasts in some cases (Zeisberg and Kalluri, 2010).

In relation to the origin of cardiac fibroblasts, there are reportedly two major sources. Firstly, cardiac fibroblasts are thought to be predominantly derived from the pro-epicardial organ (Manner et al., 2001). Cells from the pro-epicardium detach and migrate over the surface of the heart to form the epicardium (Lie-Venema et al., 2007). Subsequently epithelial cells of the epicardium undergo epithelial-mesenchymal-transition (EMT) to form mesenchymal cells that invade the developing myocardium (Manner, 1999). In addition, a subset of these mesenchymal cells acquires migratory properties and invades the developing myofascial planes to occupy an interstitial position between cardiomyocytes and to become resident cardiac fibroblasts (Gittenberger-de Groot et al., 1998; Perez-Pomares et al., 2002). It is also known that some fibroblasts may originate from progenitor stem cells present in the circulation or in the heart itself. Recent evidence has shown postnatal recruitment of circulation fibroblast progenitor cells into the ventricular myocardium (Visconti and Markwald, 2006). Another consideration is that following birth, the neonatal heart adapts to a sudden increase in systolic pressure by increasing ventricular wall thickness and tensile strength (Borg et al., 1984). This occurs as a result of a significant increase in the number of cardiac fibroblasts together with synthesis and deposition of collagen that provides contact between cardiomyocytes and fibroblasts, the fibroblasts themselves or the ECM. Thus, despite the major recognized source of cardiac fibroblasts is an epithelial layer that forms over the mid-gestation heart and is maintained in adult (Smith et al., 2011; Acharya et al., 2012), other lineages, including, pericytes, smooth muscle cells and, perhaps, myocytes and endothelium (Mikaway and Gourdie, 1996; Gittenberger-de Groot et al., 1998) also derive from epicardium. On the other hand, in contrast to cardiac fibroblasts, valvular fibroblasts are thought to be derived from the endothelium overlying the region of the atrio-ventricular valve formation (Markwald et al., 1977; Eisenberg et al., 1995). The endothelium overlying the valve leaflets undergoes endothelial-mesenchymal-transition (EndMT) to generate cardiac fibroblasts that invade the valvular mesenchyme and contribute to the collagenous structure of the valve. Finally, a small subset of fibroblasts within myocardium, mostly found in the right atrium, appear to be derived from the neural crest. These cells represents a very

heterogeneous population derived from the dorsal aspect of the neural tube and giving rise to various lineages including neurons, melanocytes and mesenchymal cells (Le Douarin et al., 2012).

The origin of fibroblasts constituting fibrotic lesions in failing hearts has been controversial. However, it is important to understand cardiac fibroblasts origin given that these cells are involved in several heart disease complications, such as myocardial fibrosis. Thus, the potential to limit or modulate their proliferation or their derivation from other cells is an attractive therapeutic option. As an example, inhibition of fibroblast formation from other cellular sources such as endothelial cells via EndMT appears even more attractive because EndMT and bone marrow-derived fibroblasts within the myocardium are exclusively associated with pathological conditions, but not present in the normal heart.

As we can see, from birth to death, heart changes in a dynamic fashion regarding its basic constituents, cell types and extracellular matrix. In fact, in the rat heart, cardiac fibroblasts constitute approximately 30% of the total number of heart cells on the first day of life. Conversely, by the 15th day of postnatal life, these cells comprise approximately 2/3 of the total numbers of cells in the rat heart (Zak, 1974; Banerjee et al., 2007). Similarly, in humans, non-myocyte cells also comprise approximately 70% of the total number of cardiac cell types (Camelliti et al., 2005). These findings support the idea that caution must be taken in interpreting data obtained in developmentally immature cells when one wants to extrapolate the results to adulthood (Porter and Turner, 2009).

In the heart, cardiac fibroblasts are arranged in sheets and strands that run in parallel with muscle fibers and maintain continuity between cells in the different layers of the myocardium (Kohl et al., 2005). Thus, cardiac fibroblasts provide a structural scaffold for cardiomyocytes, distributing mechanical forces through the cardiac tissue, and mediating electric conduction (Camelliti et al., 2005; Souders et al., 2009). Due to their abundance, their strategic location and potential for activation, cardiac fibroblasts may serve as sentinel cells that sense myocardial injury and trigger inflammatory and reparative responses (Shinde et al., 2014). Although cardiac fibroblasts are the predominant interstitial cells in normal mammalian myocardium, their function in cardiac homeostasis remains poorly understood. Cardiac fibroblasts are known to have a leading role in the synthesis

and degradation of extracellular matrix of the heart (Camelliti et al., 2005). These cells synthesize fibrillar collagen types I and III, as a precursor polypeptide that is processed subsequently to a mature form of collagen, and interstitial collagen types IV, V and VI, which are less abundant (Fan et al., 2012). However, these cells are not only responsible for extracellular matrix production, they also contribute to structural, mechanical, electrical and biochemical properties of the heart (Krenning et al., 2010). In fact, cardiac fibroblasts respond differently to environmental stimuli with differentiation into myofibroblasts, proliferation, migration, cytokines and growth factors secretion, and extracellular matrix modifications (Porter and Turner, 2009). The possibility that cardiac fibroblasts may also contribute actively to cardiac electrophysiology has emerged only recently. Cardiac fibroblasts have a high cell membrane resistance, which makes them good conductors for electrical signals (Kohl, 2003). In addition, these cells are coupled to cardiomyocytes through gap junctions containing connexin-43 and connexin-45 (Kohl, 2003) and recent in vitro evidence showed that this coupling allows for electrical signal transmission (Miragoli et al., 2006). Interconnectivity between cardiomyocytes and fibroblasts may provide synchronization of spontaneous activity in distant cardiac myocytes (Rohr, 2004). Another active contribution of cardiac fibroblasts to cardiac electrophysiology may be found as they exhibit mechano-sensory properties. Cardiac fibroblasts express multiple stretch-activated ion channels that are permeable to Na^+, K^+ and Ca^{2+} (Hu and Sachs, 1997). In response to mechanical stimuli, imposed by contractile activity of the surrounding myocardium, these ion channels open and lower fibroblast membrane potential making the fibroblast an efficient mechano-electrical transducer (Kamkin et al., 2003). In fact, despite cardiac fibroblasts are electrically non-excitable, it is important to recognize that they contain an array of ion channels, exchangers, and pumps. Examples include voltage-gated K^+ channels, chloride channels, voltage-gated proton channels, sodium/calcium exchangers, sodium/potassium ATPases, and stretch-activated channels. As an example, we may appoint to the recently described transient potential receptors (TRP) TRPM7 (Guo et al., 2014), TRPV4 (Adapala et al., 2013), and TRPC6 (Davis et al., 2012), that will be described with more details later in this manuscript.

Another consideration is that structural flexibility of fibroblasts and their ability to release soluble mediators (e.g. ATP, UTP) in response to mechanical

perturbation, associated with a favorably location in the heart, make these cells preferential in propagation/amplification of signals. As already mentioned, interactions between cardiac fibroblasts and cardiomyocytes are mostly mediated via paracrine factors such as cytokines or growth factors, direct cell-cell contacts via gap junctions or through indirect interactions via extracellular matrix proteins. Both cell types can produce and secrete a number of different chemokines, cytokines, and growth factors. In addition, ATP can also act in a paracrine manner on early fibroblast activation in ischemic hearts (Dolmatova et al., 2012). During cardiac stress, ATP is released from cardiomyocytes via pannexin 1 (Panx 1) channels which led to fibroblasts conversion into activated myofibroblasts via the activation of mitogen-activated protein kinase (MAPK) and p53 signaling pathways (Dolmatova et al., 2012). Other paracrine factors include nucleic acids such as regulatory RNA or DNA, which can act as paracrine signaling mediators between cells (Hergenreider et al., 2012; Halkein et al., 2013). In addition, recent studies demonstrated the importance of secreted exosomes for cardiovascular biology (Sluijter et al., 2014). Thus, to develop promising strategies for the treatment of heart failure it is important to consider both cell types, not separate from each other, but as a collaborative network.

Finally some considerations must be taken in what concerns cardiac fibroblasts in culture. Because fibroblasts in normal myocardium are evolved within densely packed cardiomyocytes, a direct investigation of their function *in situ* is extremely difficult. However, when interpreting results from studies with fibroblasts kept under standard two-dimensional culture conditions, it is important to know that they normally undergo a rapid phenotype switch to myofibroblasts. In fact, there is vast consensus that the rigidity of the substrate for standard two-dimensional culture systems is of core importance for phenotype switch induction. In addition to the mechanical cues offered to the adhering cells by the culture substrate, the phenotype switch is also controlled by cytokines and growth factors among which TGF-β_1 has the most prominent role. Because cultured fibroblasts secrete TGF-β_1 and simultaneously express the respective receptors, these molecules may have an autocrine effect, contributing to their own phenotype switch.

1.2.2. Cardiac fibroblasts: calcium dynamics

The calcium ion (Ca^{2+}) is an intracellular messenger that controls a wide range of cellular processes, such as gene transcription, metabolic processes, signal transduction, contraction, proliferation and differentiation (Resende et al., 2013). In most cells, Ca^{2+} has its major signaling function when it is elevated in the cytosolic compartment, where it can diffuse into organelles such as mitochondria and the nucleus. The intracellular calcium concentration increase is dependent on two mechanisms: calcium influx from the plasma membrane or release from intracellular stores, such as the endoplasmic reticulum (Patergnani et al., 2011).

In what concerns cardiac fibroblasts, the electrophysiological properties of these cells will influence their ability to exhibit excitation-secretion coupling, fibroblasts-cardiomyocytes coupling as well as fibroblast-fibroblast interactions (Yue et al., 2013). Despite it is not fully understood which channels determine resting membrane potential in cardiac fibroblasts, it was reported that the resting membrane potential of cardiac fibroblasts *in situ* is between -31 and -16 mV (Kamkin et al., 1999; 2003). Thus, it is less negative than that of cardiomyocytes (roughly -70 to -80 mV, for atrial cardiomyocytes).

Now, we will briefly describe some findings on calcium dynamics of cardiac fibroblasts. In 1996, Kiseleva and colleagues demonstrated, for the first time, the importance of Ca^{2+} in fibroblasts biology. Their work demonstrated that Ca^{2+} entry through stretch-activated channels located in the plasma membrane and Ca^{2+} release from the endoplasmic reticulum play key roles in the generation of a mechanical-induced-potential in rat cardiac fibroblasts (Kiseleva et al., 1996). Other studies demonstrated that Ca^{2+} signals are essential for diverse cellular functions including differentiation, gene expression, cell proliferation, growth, and death (Berridge et al., 2003). For instance, in rat cardiac fibroblasts, chelating external Ca^{2+} by EGTA has shown to prevent substance P-induced proliferation (Shivakumar et al., 2001). In addition, it has been also described that intracellular Ca^{2+} changes contribute to AngII-induced proliferation of cardiac fibroblasts (Olson et al., 2008).

Some authors argued that cardiac fibroblasts lack voltage-gated Ca^{2+} channels (Kohl and Noble, 1996). However, in what concerns L-type Ca^{2+} channels, this issue is at least controversial. Some authors claim that cardiac fibroblasts do not exhibit L-type Ca^{2+} currents (Kohl and Noble, 1996; Kamkin et al.,

1999), while others demonstrate the existence of L-type calcium channels and their involvement on TGF-β1- and Smad2-dependent protein synthesis in these cells (Lei et al., 2011). Moreover, an *in vivo* study showed that mibefradil, a non-selective L- and T-type Ca^{2+}-channel blocker, reduced collagen production and fibroblast differentiation in rats treated with AngII or aldosterone (Ramires et al., 1998).

Another important consideration is the existence of the previously mentioned TRP channels in cardiac fibroblasts. TRP channels are responsible for Ca^{2+} entry in various cell types (Montell, 2005; Nilius, 2007) and they are not voltage-gated channels, but are activated by a variety of stimuli including membrane-receptor stimulation, oxidative stress, mechanical stretch, cell-metabolite accumulation, and thermal or sensory stimuli (Montell, 2005; Nilius, 2007). In fact, TRP channels are highly expressed in the heart. In what concerns cardiac fibroblasts, TRPC1-3 and TRPC5-7 mRNA were detected in rat species by RT-PCR, and the presence of TRPM7-like (Runnels et al., 2002) and TRPV4 currents were demonstrated by whole-cell current recordings. Nishida and colleagues reported the presence of TRPC1, TRPC3, TRPC6, and TRPC7 in rat cardiac fibroblasts (Nishida et al., 2007). In cultured human cardiac fibroblasts, TRPC1, TRPC4, and TRPC6 were found to be abundantly expressed at mRNA levels (Chen et al., 2010). TRPC1, TRPC4, TRPC6, TRPV2, TRPV4, TRPM4, and TRPM7 mRNA are expressed in human atrial fibroblasts (Du et al., 2010). These channels have been involved in numerous cellular functions and in the pathophysiology of many diseases in the cardiovascular systems and, thus, may become potential therapeutic targets.

1.2.3. Cardiac fibroblasts as therapeutic targets

Despite the numerous signaling pathways implicated in the activation of cardiac fibroblasts and in the pathological remodeling of the heart, currently exist limited clinical intervention that effectively target this cell type and its pathological contributions to disease progression. On the other hand, the actions of certain pharmacological approaches may be mediated, at least in part, through effects on fibroblasts. For example, the treatment with angiotensin-converting enzyme inhibitors and AT1 receptor blockers reduces mortality and has protective effects

on the development of heart failure. Despite, the classical described action mechanism not include, usually, the effects on cardiac fibroblasts population, we learned that these effects are associated with attenuated cardiac fibrosis. In addition, aldosterone antagonism in patients with acute anterior myocardial infarction attenuates remodeling, reducing plasma levels of pro-fibrotic markers. However, because these approaches have multiple other effects on different cell types, the extent to which their anti-fibrotic actions are responsible for the observed clinical benefits is unknown.

As mentioned early, cardiac fibroblasts have an important role on structure and function of the myocardium contributing to structural, biochemical, mechanical and electrical characteristics of cardiac function (Camelli et al., 2005). Because the absence of contractile capacity on cardiac fibroblasts, these cells are underestimated. However, their placement outside the contractile machinery can be an advantage. Thus, cardiac fibroblasts would offer a different modality of mechano-receptive signals and, by direct gap-junctional coupling with other fibroblasts and/or adjacent cardiomyocytes, have the potential to affect electrophysiology. Therefore, it is important to remember that the modulation of these cells may present potentially novel therapeutic targets or strategies for the treatment of progressive heart diseases.

Despite countless findings suggesting novel pathways of physiological and pathophysiological modulation of myocardial performance by cardiac fibroblasts, several important details in these mechanisms are still missing. The knowledge about the signaling mechanisms that regulate cardiac fibroblast functions, the distinctive properties of these cells, the ability to integrate many stimuli through receptor-specific signaling pathways as well as the diversity of endpoint responses, may provide very important insights to the development of new drugs for the treatment of heart diseases.

In addition, another area of increasing research is the study of mechanisms underlying intercellular communication in normal and failing hearts. As mentioned above, the homeostatic maintenance of heart physiology and/or remodeling processes involve intercellular communication between cardiomyocytes, fibroblasts, endothelial or immune cells. Thus, modulation of these processes by intervention at different stages of these mechanisms may also contribute to novel therapeutic approaches.

1.3. Purinergic signaling

1.3.1. Purinergic signaling: an overview

The term ‘purinergic signaling’ was coined for the first time by Geoffrey Burnstock who described the finding that the principal active substrate released from nerves was the purine nucleotide adenosine triphosphate (ATP) (reviewed by Abbracchio et al., 2009). In fact, until then, ATP was commonly conceptualized as the currency of intracellular energy metabolism as well as a component of nucleic acids. Since then, nucleotide signaling was found to be involved in many different cell types and tissues and it was established a role for ATP outside the cell as an intercellular messenger as well (Hattori and Gouaux, 2012). After these findings, it was demonstrated that ATP may act either as a co-transmitter or a sole transmitter in most nerves in the peripheral and central nervous systems (Abbracchio et al., 2009; Burnstock et al., 2011).

Intracellular ATP is synthesized via complex mechanisms to maintain or rapidly restore the intracellular level (Gordon, 1986). Thus, intracellular ATP concentrations in eukaryotic cells are high (3–10 mM) and it can be concentrated at even higher concentrations (up to 100 mM) inside synaptic vesicles (Burnstock, 2007) compared to very low physiological extracellular concentrations (400–700 nM) (Dubyak, 1991; Dubyak and El-Moatassin, 1993; Bours et al., 2006). Consequently, in normal resting conditions, there is a constant efflux of ATP, which is balanced by its extracellular enzymatic breakdown (Schwiebert, 1999; Lazarowski et al., 2003). The low concentration is achieved by several mechanisms like minimal permeation of cytosolic ATP across lipid bilayers, and due to ubiquitous ectoenzymes that hydrolyze extracellular nucleotides. This ensures that putative signaling agents, like ATP, are only transiently present and only in response to specific physiological and/or pathological situations (Dubyak and El-Moatassin, 1993; Dubyak, 1991). Under these situations, ATP may be release by several mechanisms that will be discussed later. Thus, once released into extracellular medium, these low micromolar ATP concentrations can affect a variety of biological processes in various cells and tissues (Gordon, 1986; Dubyak and El-Moatassin, 1993), like platelet aggregation, neurotransmission, cardiac function and muscle contraction (Gordon, 1986).

Extracellular ATP exerts its effects by binding to purinergic receptors, which were first defined in 1976 (Burnstock, 1976). Several studies demonstrated that cells are able to release purines and pyrimidines to the extracellular medium. In fact, extracellular nucleotides have been implicated in a wide range of biological processes as smooth muscle contraction, inflammation, platelet aggregation and pain, but also, which is most relevant to this study, in the fibrosis process (Burstock, 2006). Despite the fact that the potent biological action of ATP on most cell types is well established, the source of extracellular ATP, the mechanism by which cells release the nucleotide, the biochemical mechanisms regulating the amount of these molecules within the heart or even the cell types involved are still a matter of debate and also inclined for pharmacological manipulation.

An important consideration to take into account is that purinergic signaling is not only a set of ligands and receptors, but a complex and dynamic system consisting of nucleotide channels and transporters, enzymes and purinoceptors, which are designated by the term 'purinome' (Volonté and D'Ambrosi, 2009). Each component of the system will be explained in detail later on. The complex cooperation between the different constituents of the purinome enables the transfer of information and its extracellular processing contributes as a whole (not alone) for triggering, maintaining and closing of purinergic signaling. This coordination between the various compartments leads to the activation of specific receptors, ending their signaling pathways by enzymatic metabolism or uptake systems (Volonté and D'Ambrosi, 2009). Thus, with the purinome concept it becomes evident that cooperation and molecular dynamics are responsible for several physiological mechanisms. Changes on the global and dynamic balance can be responsible for the emergence or spread of disease states (Volonté and D'Ambrosi, 2009).

1.3.2. Purines and pyrimidines: releasing pathways

The release of endogenous nucleotides represents a critical component for initiating a signaling cascade and it may occur by several mechanisms. Massive escape of nucleotides might occur upon cell lysis, however this nonspecific mechanism is restricted to organ injury, traumatic shock or certain inflammatory conditions (Bours et al., 2006). Non-lytic mechanisms of nucleotide efflux represent a distinct and important route of nucleotide appearance in the

extracellular milieu (Figure 1A). On other hand, it is also known that various excitatory/secretory tissues store purines, like ATP and ADP, together with other neurotransmitters and extracellular mediators, in specialized granules, and regulate the release of nucleotide-containing vesicles in a Ca^{2+}-dependent manner via regulated exocytosis (reviewed by Yegutkin, 2008) (Figure 1D). Moreover, various cells release low nanomolar concentrations of ATP at certain basal rates (Lazarowski et al., 2000; Abbracchio et al., 2006) and distinctive mechanisms could underlie constitutive versus stress-stimulated nucleotide release. The diversity of conditions in which the cells release nucleotides suggests the implication of multiple nucleotide releasing pathways. The proposed cellular mechanisms might include electrodiffusional movement through membrane ion channels, including connexin hemichannels, stretch- and voltage activated channels (Figure 1B); facilitated diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters (Figure 1C), and cargo-vesicle trafficking and exocytotic granule secretion (Figure 1D).

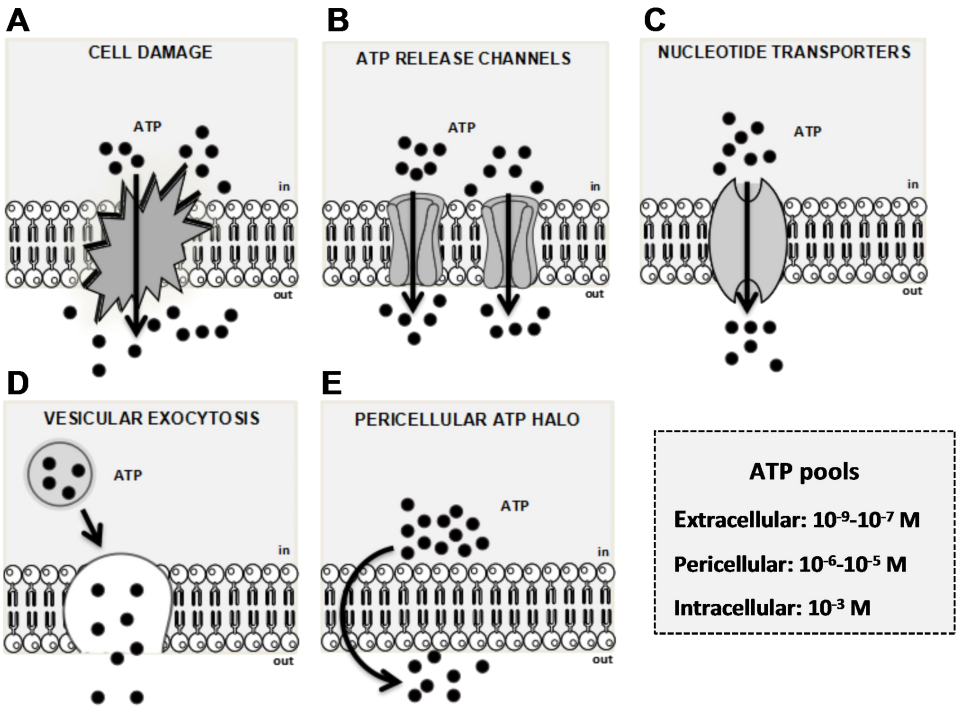

Figure 1 - Schematic representation of different mechanisms of ATP release: (A) Cell damage; (B) ATP release channels; (C) Nucleotide transporters; (D) Vesicular exocytosis; (E) Pericellular ATP halo.

1.3.3. Purines and pyrimidines: metabolic pathways

Like all signaling pathways, purinergic signaling requires mechanisms that can inactivate the signaling cascade (Kegel et al., 1997; Zimmermann, 2000) and define the endpoint of response. Thus, to remove or inactivate extracellular signaling molecules, they can be taken up by the cell or be hydrolyzed by ectonucleotidases. In addition, purinergic receptors can also be desensitized or down regulated contributing to the termination of response.

After released, ATP and the other nucleotides may be hydrolyzed extracellularly by a cascade of plasma membrane-bound enzymes - ectonucleotidases - resulting in the formation of the respective nucleoside and free phosphates. The cleaved off phosphate can be recycled and used for nucleotide resynthesis and the nucleosides can be taken up by the cell via specific transporters and rephosphorylated intracellularly (Zimmerman et al., 2000). In addition to the role of ectonucleotidases in the control of ATP (and UTP) responses, this pathway can lead to the production of biologically-active derivatives as well (Zimmermann, 2000).

In this context, in addition to ADP and ATP, another important molecule, adenosine, appears in the extracellular milieu either via direct release via nucleoside transporters or as result of the breakdown of ATP/ADP via the ectonucleotidase cascade. In fact, the extent of the paracrine activity mediated by ATP/UTP (and related purines and pyrimidines) may be limited by the presence of ectonucleotidases, which sequentially degrade nucleoside 5'-triphosphates to their respective nucleoside 5'-di- and monophosphates, nucleosides and free phosphates or pyrophosphate (Robson et al., 2006). These enzymes have distinct biochemical properties.

According to substrate specificity and products formation, ectonucleotidases are divided into four major families (Abbracchio et al., 2009): ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase and/or phosphodiesterases (E-NPPs), alkaline phosphatases, and ecto-5'nucleotidase (CD73). E-NTPDases and E-NPPs hydrolyze ATP and ADP. In particular, E-NTPDase1 hydrolyzes ATP and ADP equally well, E-NTPDase2 preferentially hydrolyzes triphophonucleosides, and E-NTPDase3 has an intermediate hydrolysis profile (Kukulski et al., 2005). The hydrolysis of tri- and diphosphonucleosides by NTPDases yields AMP as final products that can be fully

dephosphorylated to adenosine by ecto-5'-nucleotidase (Colgan et al., 2006). Alkaline phosphatases equally hydrolyze nucleoside tri-, di- and monophosphates. In what concerns dinucleoside polyphosphates, nicotinamide adenine nucleotide (NAD^+) and uridine diphosphate/UDP sugars, are substrates exclusively for E-NPPs (Abbracchio et al., 2009).

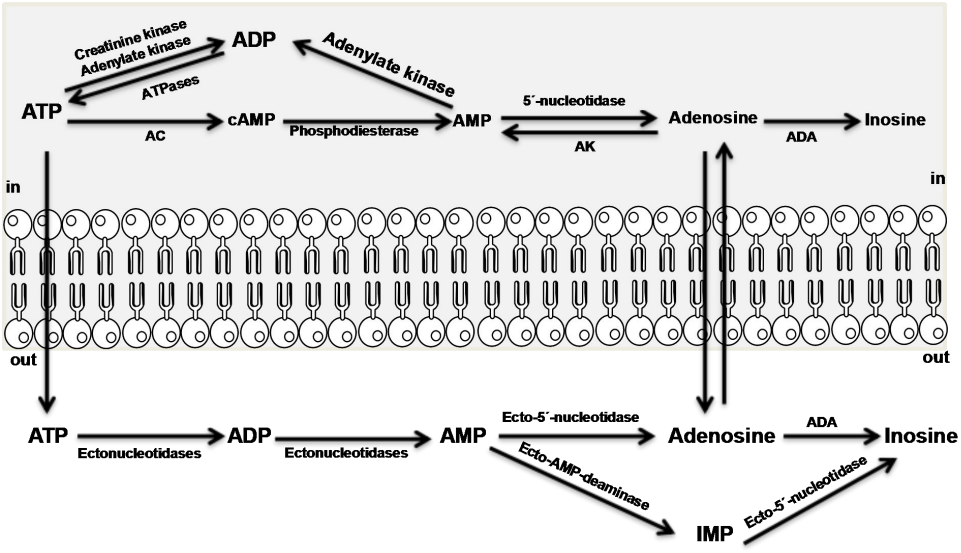

Figure 2 - Schematic representation of ATP metabolism, inside and outside of the cell.

Furthermore, uracil nucleotides (UTP and UDP) can also be released as extracellular signaling molecules. It has been described that various cells types release low nanomolar UTP concentrations (reviewed by Yegutkin, 2008). Moreover, studies with murine airway epithelial cells transfected with the $P2Y_4$ receptor also demonstrated significant UTP release from mechanically stimulated cells, which, in turn, contributes to the generation of Ca^{2+} waves and coordination of the integrated epithelial stress response (Homolya et al., 2000). Thus, all these molecules, extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP), are important signaling molecules that mediate diverse biological effects through the activation of purinergic receptors that will de described in the next section.

1.3.4. Purinergic receptors

As already mentioned, purinergic receptors were first described by G. Burstock in 1976 and they can now be categorized into two major groups: those

responding preferentially to adenosine (P1 receptors) and those responding to ATP/UTP/ADP/UDP (P2 receptors) (Burnstock, 2006) (Figure 3).

There are four types of membrane-bound adenosine P1 receptors: A_1, A_{2A}, A_{2B} and A_3 receptors, each encoded by separate gene and with different functions, despite some common characteristics.

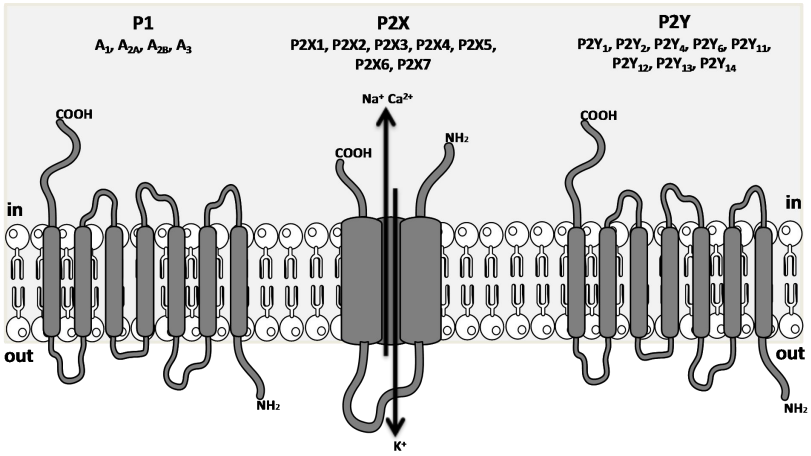

Figure 3 - Schematic representation of the three different families of purinergic receptors: metabotropic P1 receptors, ionotropic P2X receptors and metabotropic P2Y receptors.

P1 receptors are G-protein coupled receptors (GPCR) composed of seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus (Figure 3). The A_1 and A_3 receptors couple to G_i protein and inhibit adenylate cyclase, leading to a decrease in cellular cAMP levels, while A_{2A} and A_{2B} receptors couple to G_s proteins, stimulate adenylate cyclase and consequently leading to an increase in cellular cAMP levels (Moro et al., 2006). Additionally, to the adenylate cyclase coupling, all four subtypes may positively couple to phospholipase C via different G protein subunits (Moro et al., 2006). The primary and secondary transduction mechanisms as well as the main agonists and antagonists of the receptors are summarized in the Table 1.

Table 1 - Characteristics of P1 receptors.

Receptor	Endogenous ligand	Transduction mechanisms	Agonists	Antagonists
A_1	Adenosine	**Primary:** $G_i/G_o \rightarrow$ AC inhibition **Secondary:** $G_s \rightarrow$ AC stimulation $G_q/G_{11} \rightarrow$ PLC stimulation	Adenosine, CCPA, R-PIA, NECA	DPCPX, CGS15943, CPT, FR194921, WRC-0571, XAC, N-040, MRS1754
A_{2A}		**Primary:** $G_s \rightarrow$ AC stimulation **Secondary:** $G_q/G_{11} \rightarrow$ AC stimulation and PLC activation	Adenosine, ATL146e, CGS21680, CV-1808, IAB-MECA, NECA	CGS15943, MRS1093, SCH58261, ZM241385, KF17837
A_{2B}		**Primary:** $G_s \rightarrow$ AC stimulation **Secondary:** $G_q/G_{11} \rightarrow$ PLC activation	Adenosine, NECA	AS99, AS101, MRE2029-F20, MRS17541, OSIP339391, XAC Enprofylline
A_3		**Primary:** $G_i/G_o \rightarrow$ AC inhibition **Secondary:** $G_i/G_o \rightarrow$ PLC stimulation	Adenosine, I-ABA, IAB MECA, IB-MECA, NECA, 2-CI-IB-MECA	MRS1177, MRS1220 MRE3010F20, VUF8504, L-268605, MRS1191, MRS1523

Adapted from IUPHAR and Burnstock (2007)

The P2 receptors were subdivided into two major families, a P2Y family of G protein-coupled receptors and a P2X family of ligand-gated ion channel receptors, based on their mechanism of action, pharmacology and molecular cloning (Ralevic and Burnstock, 1998; Abbracchio et al., 2009). Currently, seven subtypes of the P2X family and eight subtypes of the P2Y family have been cloned and functionally characterized (reviewed by Burnstock, 2006).

Members of the existing family of ionotropic P2X1–7 receptors are designated according to the historical order of cloning and show the following: intracellular N- and C-termini, two transmembrane domains, a large extracellular loop, with 10 conserved cysteine residues forming a series of dissulphide bridges and an ATP-binding site, which may involve regions of the extracellular loop adjacent to transmembrane domains.

The transduction mechanisms as well as the main agonists and antagonists of the receptors are summarized in the Table 2.

Table 2 - Characteristics of P2X receptors.

Receptor	Endogenous ligand	Transduction mechanisms	Agonists	Antagonist
P2X1	ATP	Ion channel (Ca^{2+} and Na^{+})	ATP,α,β-MeATP, 2-MeSATP	IP_5I, NF023, NF449, TNP- ATP
P2X2		Ion channel (mainly Ca^{2+})	ATP, ATPγS	isoPPADS, NF279, NF770, RB-2, suramin
P2X3		Intrinsic ion channel	ATP, 2-MeSATP	A317491, IP_5I, NF110, PPADS, TNP-ATP, RO-85
P2X4		Ion channel (mainly Ca^{2+})	ATP, α,β-MeATP, Ivermectin potentiation	Coomassie blue, TNP-ATP 5-BDBD, BBG
P2X5		Intrinsic ion channel (Cl^-)	ATP, α,β-MeATP, 2-MeSATP, ATPyS	Coomassie blue, PPADS, suramin, BBG
P2X6		Intrinsic ion channel	-	-
P2X7		Intrinsic ion channel and a pore	ATP, BzATP	KN62, KN04, MRS2427, O-ATP, A-438079, A-740003

Adapted from IUPHAR and Burnstock (2007)

The P2Y receptors also belong to the GPCR family and share a common topology: seven transmembrane domains and an extracellular N-terminus and an intracellular C-terminus (Figure 3). These receptors show a high level of sequence homology between some transmembrane domains but, on the other hand, a structural diversity of intracellular loops and C-terminus among P2Y subtypes, thus influencing the degree of coupling with $G_{q/11}$, G_s and G_i proteins. Each P2Y receptor binds to a single heterotrimeric G protein (typically $G_{q/11}$), although $P2Y_{11}$ can couple to both $G_{q/11}$ and G_s, whereas $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ couple to G_i proteins P2Y receptors may form homo- and heteromultimeric assemblies under some conditions, and many cells express multiple P2Y receptor subtypes.

Some P2Y receptors are activated mainly by nucleoside diphosphates ($P2Y_{1,6,12,13}$), while others are activated mainly by nucleoside triphosphates ($P2Y_{2,4}$). Some P2Y receptors are activated by both purine and pyrimidine nucleotides ($P2Y_{2,4,6,11}$), and others by purine nucleotides alone ($P2Y_{1,12,13}$). In response to nucleotide activation, recombinant P2Y receptors either activate phospholipase C and release intracellular calcium or affect adenylyl cyclase and alter cAMP levels. Besides the described characteristics of purinergic receptors, it is important to note that the pharmacological properties of nucleotides may be different depending on the species activating different signaling pathways.

The primary and secondary transduction mechanisms as well as the main agonists and antagonists of the receptors are summarized in the Table 3.

Table 3 - Characteristics of P2Y receptors.

Receptor	Endogenous ligand	Transduction mechanisms	Main agonists	Main antagonist
$P2Y_1$	ADP>ATP	**Primary:** G_q/G_{11} → PLC activation **Secondary:** G_i/G_o family → Ion channel (K^+ and Ca^{2+})	ADP, 2-MeSADP, ATP, ATPγS MRS2365, ADPβS	2-ClATP, 2-MeSATP, MRS2179, MRS2279, MRS2500
$P2Y_2$	UTP=ATP	**Primary:** G_q/G_{11}→ PLC activation **Secondary:** G_i/G_o family; G_{12}/G_{13} family	ATP, Ap_4A, UTP, UTPγS, 2-thio-UTP	ARC126312, suramin, RB-2
$P2Y_4$	UTP>ATP	**Primary:** G_q/G_{11} → PLC activation **Secondary:** PLA_2 stimulacion	ATP, Up_4U, Up_4dC, UTP	PPADS, RB-2, suramin
$P2Y_6$	UDP>>UTP> ATP	**Primary:** G_q/G_{11}→ PLC activation **Secondary:** G_{12}/G_{13} → AC stimulation	MRS2693, UDP, UDPβS, Up_3U, 3-Phenacyl-UDP	MRS2567, MRS2575, MRS2578, RB-2
$P2Y_{11}$	ATP>UTP	**Primary:** G_q/G_{11} → PLC activation **Secondary:** G_S→AC stimulation	ARC67085, ATP, ATPγS, BzATP,dATP	5'AMPS, NF157, NF340, RB-2, suramin
$P2Y_{12}$	ADP>>ATP	**Primary:** G_i/G_o→ AC inhibition **Secondary:** Gi/G_o→ AC inhibition	ADP, ADPβS, 2-MeSADP, 2-MeSATP	AZD6140, BX667, Cangrelor, Clopidogrel, INS50589, AR-C66096, CT50547, PSB0413, ARL66096
$P2Y_{13}$	ADP>>ATP	**Primary:** G_i/G_o→ AC inhibition	ADP, ADPβS, 2-MeSADP, 2-MeSATP	Ap_4A, Cangrelor, 2-MeSAMP, MRS2211, MRS2603, RB-2
$P2Y_{14}$	UDP-glucose	**Primary:** G_i/G_o→ AC inhibition **Secondary:** G_i/G_o→ PLC stimulation	UDP, UDP-galactose, UDP-glucuronate, UDP- glucose	UDP

Adapted from IUPHAR and Burnstock (2007)

1.4. Purinergic signaling in the heart

1.4.1. Purinergic signaling in the heart: an overview

Since the pioneering work of Drury and Szent-Gyorgyi (1929) on the effects of the adenine compounds in the heart (Drury and Szent-Gyorgyi, 1929), an ever increasing number of studies have been published in this area. Over the years, various aspects of purinergic signaling have been issued, including: physiological

roles of cardiac P2X and P2Y receptors, roles of adenosine in health and disease, effects of ATP and adenosine on coronary myocytes and purine degradation pathways in the myocardium (reviewed by Burnstock and Pelleg, 2015). Despite the existence of all these works, there is still a lack of information on the purinergic signalling mechanisms at cellular and molecular levels.

In the cardiac territory, cardiomyocytes, endothelial cell, smooth muscle cells and fibroblasts may all release nucleotides (purines and pyrimidins) that, under pathological situations, may assume particular relevance (Shryock and Belardinelli, 1997). In fact, these released nucleotides may work as autocrine and/or paracrine mediators leading to activation of surrounding cells via many subtypes of purinergic receptors (Burnstock et al., 2006). Questions still remain about the signaling mechanisms involved in the release, maintenance and termination of the signal.

Concerning the discoveries about purinergic signaling in the heart, one may emphasize the effect of ATP in heart's physiology. It is well established that there are several sources of ATP in the heart, both in physiological and pathological conditions. Despite ATP and other adenine compounds are often stored with sympathetic amines in nerve endings (Meldrum and Burnstock, 1983), several studies demonstrated that these nucleotides may also come from ischemic cardiomyocytes, erythrocytes, platelets, apoptotic cells, inflammatory cells, smooth muscle cells or endothelial cells (Forrester and Williams, 1977; Pearson and Gordon, 1979; Clemens and Forrester, 1981; Fredholm et al., 1982; Gordon, 1986; Vial et al., 1987; Christie et al., 1992; Vassort, 2001). Some authors suggested that ATP released from nerve terminals appears to be functionally important in the pacemaker region because some the effects of sympathetic nerve stimulation, including tachycardia, can be reproduced by ATP application. In addition, the early stage of tachycardia can be deleted after P2 receptors desensitization (Bramich et al., 1990). Thus, ATP released from sympathetic nerves can increase cardiac contractility acting in an additive or synergistic fashion with β_1 receptors. Several effects of ATP in the cardiovascular system were described: negative or positive inotropic effects depending on species, negative chronotropic and dromotropic effect and anti-hypertrophic myocardial effect (Vassort, 2001).

Given its more widespread clinical application another relevant aspect of purinergic signaling in the heart concerns adenosine. It is known that intravenous

administration of this nucleoside rapidly slowdown the sinus rhythm (negative chronotropism), decreases atrioventricular conduction (negative dromotropism), reduces the contractile force of the myocardium (negative inotropism), and dilates the coronary vessels (Drury and Szent-Gyorgyyi, 1929). Adenosine is a "chronoselective" atrial depressant, because its bradicardic action is observed in concentrations below those required to decrease inotropism. This may be of significant clinical advantage to prevent cardiac stunning during cardioversion of paroxysmal supraventricular tachyarrhythmias by adenosine or its stable analogues (e.g. tecadenoson). Elucidation of the mechanisms responsible for adenosine A_1 receptor-mediated "chronoselectivity" was recently undertaken by our group using the spontaneously beating rat atria (Bragança et al., 2016). Adenosine decreases SA node automatism mainly by promoting K^+ efflux through βγ subunits of G protein-coupled inwardly rectifying GIRK/KIR3 channels. This effect, may be counteracted by inhibition of $K_{Ca}2$/SK currents (probably via G protein α subunit) leading to a subsequent prolongation of atrial repolarization. The increase in the time available for Ca^{2+} influx through voltage-sensitive Ca_v1 (L-type) channels seems to be crucial to sustain inotropy in the presence of adenosine in concentrations causing significant negative chronotropic actions (Bragança et al., 2016).

Finally, it is also worth to mention the role of uracil nucleotides in the heart. Like ATP, uracil nucleotides also have a positive inotropic effect (Froldi et al., 1994; Froldi et al., 1997; Podrasky et al., 1997; Froldi et al., 2001). On the other hand, UTP causes hypertrophy of neonatal cardiomyocytes (Pham et al., 2003), and this unlike the ATP effect which inhibits hypertrophy inducing cellular apoptosis and necrosis (Zheng et al., 1996).

1.4.2. Purinergic signaling in the heart: cardiac fibroblasts

Numerous studies confirmed that cardiac fibroblasts express P2 receptors and extracellular nucleotides could modulate several functions of these cells. Table 4 summarizes the work around P2 receptors in these cells. Data so far obtained strengthen the presence of P2 receptors in cardiac fibroblasts. However, there is little information in the literature in what concerns the activity of purinergic receptors in these cells.

The most described effect of purines in cardiac fibroblasts is the anti-fibrotic and anti-proliferative effect of adenosine (Epperson et al., 2009; Lu et al, 2013). It was found that adenosine, acting through A_{2B} receptors, inhibits collagen and protein synthesis in cardiac fibroblasts (Dubey et al., 1998). Evidences coming from different studies show that A_{2B} receptors agonists might protect against cardiac fibrosis. Other evidences demonstrated that elevated glucose levels increases A_1 and A_{2A} receptors expression, decreased expression of A_3 receptors, but had no effect on A_{2B} receptors expression in cardiac fibroblasts (Grden et al., 2006). Another study using RT-PCR revealed that mRNA for all four P1 receptors subtypes, A_1, A_{2A}, A_{2B} and A_3, are expressed in rat cardiac fibroblasts, with a prevalence of A_2 receptors, acting via cAMP second messenger (Epperson et al., 2009). In primary cultures of adult rat cardiac fibroblasts, adenosine activated A_2 receptors coupled to G_s-protein and adenylyl cyclase pathway; the resultant cAMP reduced collagen synthesis via a PKA-independent, EPAC-dependent pathway that feeds through PI3K (Villarreal et al., 2009).

Despite the existence of several studies describing the effects of adenosine in the heart, in what concerns P2 receptors there is little information. Zheng and collaborators claimed that P2Y receptors promote activation of c-fos gene expression and inhibit DNA synthesis in cultured rat cardiac fibroblasts (Zheng et al., 1998). Notwithstanding this, there is a consensus in the literature indicating that ATP, as well as UTP, transiently increases cardiac fibroblasts proliferation via the activation of $P2Y_2$ receptors, both in rats (Braun et al., 2010) and in humans (Chen et al., 2012): these findings suggest that antagonists of the $P2Y_2$ receptor may provide a mean to reduce cardiac fibrosis under pathological conditions. This receptor subtype has also been involved on Ca^{2+} signaling in human valvular myofibroblasts (Liang et al., 2008). Previous studies also demonstrated that ATP activates ionotropic P2X(4/7) receptors and up-regulates the proliferation of human cardiac fibroblasts by promoting cell cycling progression, in addition to the previous implicated $P2Y_2$ receptors (Chen et al., 2012). The existence of other purinoceptors subtypes has been evidenced, but, so far, their roles in cardiac fibroblast cells remain uncertain. For instance, Tansilla and collaborators described that $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors are co-expressed in neonatal rat cardiac myofibroblasts (Talasilla et al., 2009), but no evidences have been produced regarding their role in these cells. Using reverse

transcription-polymerase chain reaction (RT-PCR) and Northern blotting other authors demonstrated the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor transcripts in neonatal rat cardiac fibroblasts (Zheng et al., 1998). The presence of the $P2Y_{13}$ receptor was also reported in neonatal rat cardiac myofibroblasts (Talasila et al., 2009). Despite these findings, little information has been produced regarding the role of all these receptors in cardiac fibroblasts and whether their activity remains in adulthood.

It, thus, appears that purines may play a dual role in cardiac fibrosis through direct activation ATP/ADP-sensitive P2 receptors exerting a pro-fibrotic activity, which may be balanced by the indirect anti-fibrotic effect of adenosine via A_{2B} receptors (Lu and Insel, 2013). Another important issue regarding purinergic signaling in cardiac fibroblasts is the preferential coupling of these receptors to $G_{q/11}$ and G_s proteins leading to activation of phospholipase C and adenyly cyclase pathways, respectively, but few reports demonstrate their coupling to the G_i subtype (Meszaros et al., 2000). These authors claim that the expression of receptors preferentially coupled to $G_{q/11}$-, but not to G_i-linked responses, may be a way to differentiate signals affecting cardiac fibroblasts from those acting in cardiomyocytes. Moreover, it has been demonstrated that agonists that stimulate $G_{q/11}$-protein-dependent pathways in cardiac fibroblasts synergize with G_s-linked mechanisms, but this does not occur in adult cardiomyocytes (Meszaros et al., 2000). Thus, G protein-mediated signaling in cardiac fibroblasts has unique characteristics and may contribute to increase specificity of drug actions on individual cell types within the heart.

Table 4 - Purinergic receptors expression by cardiac fibroblasts

P1	**A_1**	rt	-	Gorden et al., 2006; Epperson et al., 2009
	A_{2a}	rt	-	Gorden et al., 2006; Epperson et al., 2009
	A_{2b}	rt	↑ Proliferation	Gorden et al., 2006; Epperson et al., 2009
	A_3	rt	-	Gorden et al., 2006; Epperson et al., 2009
P2X	**P2X4**	hu	↑ Proliferation	Chen et al., 2012
	P2X6	hu, pig	-	Banfi et al., 2005;
	P2X7	hu	↑ Proliferation	Chen et al., 2012
P2Y	**$P2Y_1$**	rt	-	Tansilla et al., 2009; Zheng et al., 1998; Braun et al., 2010
	$P2Y_2$	rt, hu	↑ Proliferation; Ca^{2+} signallig; regulation of angiotensin-II induced inflammatory response	Braun et al., 2010; Chen et al., 2012; Liang et al., 2008; Tansilla et al., 2009; Zheng et al., 1998; Nishidaa et al., 2011;
	$P2Y_4$	rt	-	Tansilla et al., 2009; Zheng et al., 1998
	$P2Y_6$	rt	-	Tansilla et al., 2009; Zheng et al., 1998; Braun et al., 2010
	$P2Y_{11}$	rt	-	Tansilla et al., 2009
	$P2Y_{13}$	rt	-	Talasila et al., 2009

The findings assembled in Table 4 illustrate that although almost all P1 and P2 purinoceptor subtypes seem to be present on cardiac fibroblasts, there is a lack of information in what concerns their function in these cells. We will try to fill this gap in our knowledge in the present work by focusing our efforts on the role of metabotropic P2Y purinoceptors on Ca^{2+} signaling and proliferation / type I collagen production by fibroblasts from the ventricles of adult rats.

1.5. Clinical relevance of study

As previously mentioned, heart failure is ultimately linked to myocardial fibrosis that is characterized, among other events, by dedifferentiation of cardiomyocytes and their substitution by fibroblasts leading to excess deposition of ECM proteins (Cohn et al., 2000). These cardiac remodeling events lead to distorted organ architecture and altered hemodynamic events.

Another relevant issue to the functional repercussions of myocardial fibrosis is the contribution of cardiac fibroblasts to heart electrophysiology, as emerged very recently. These cells have a high cell membrane resistance, which makes them good conductors for electrical signals (Kohl, 2003) and, on the other hand, they are coupled to cardiomyocytes through gap junctions containing connexins-43 and connexin-45 (Kohl, 2003). In fact, many studies point towards an interaction between cardiomyocytes and fibroblasts that may provide

synchronization of spontaneous electrical activity in distant cardiomyocytes (Rohr, 2004). These findings support the concept that cardiac fibroblasts are important on both heart's physiology and pathophysiology, offering the possibility of these cells being suitable drug targets for the treatment of various cardiac disorders.

Another interesting consideration is that purinergic signaling may potentially play a role on cardiac fibroblasts activity, transdifferentiation into myofibroblasts, intercellular cells communication and, subsequent, cardiac remodeling. Thus, a better understanding of the factors that regulate cardiomyocytes signaling to surrounding fibroblast and *vice versa* may provide new insights into the development of cardiac fibrosis and will permit identification of novel therapeutic targets for its management. As already mentioned, ATP and UTP are released in huge amounts during myocardial infarction in humans and these, together with some of their extracellular metabolites (*e.g.* ADP, UDP and adenosine), might play putative important roles in myocardial remodeling through the activation of purinoceptors in cardiac cells, both cardiomyocytes and cardiac fibroblasts. Despite our knowledge on the multiple roles of endogenous nucleotides and nucleosides on cardiac remodeling, further studies are required before manipulation of these signaling molecules becomes accepted as a useful therapeutic strategy for the treatment of heart diseases.

In this context, this study was designed to investigate the purinergic signaling mechanisms involved in cell activation, proliferation and differentiation of cardiac fibroblasts from adult rat ventricles and to clarify the underlying intracellular molecular pathways responsible for P2Y receptor responses in these cells.

CHAPTER 2. GOALS

Given the data from the literature reviewed in the previous chapter, we aimed at investigating the purinergic signaling mechanisms involved in the proliferation and differentiation of cardiac fibroblasts from ventricles of adult rats in order to discover novel therapeutic targets to attenuate / prevent cardiac remodeling.

The specific aims of the project were:

1. To investigate, the effect of uracil nucleotides on $[Ca^{2+}]_i$ signaling and long-term growth of cultured cardiac fibroblasts from adult rat ventricles in order to find novel targets for therapeutic intervention in cardiac remodeling. In addition, we attempted to investigate the intracellular signaling pathways implicated in $[Ca^{2+}]_i$ oscillations and proliferation of cardiac fibroblasts due to activation of uracil nucleotides-sensitive P2Y receptors ($P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$).
2. Likewise, we also attempted to fill a gap in our knowledge regarding the role of the ATP intermediate metabolite, ADP, on $[Ca^{2+}]_i$ signaling and proliferation of cardiac fibroblasts mediated by ADP-sensitive $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors.
3. G-protein coupled receptors, like all P2Y receptor subtypes, may (1) assemble to membrane delimited pathways (e.g. ion channels, transporters, enzymes) within lipd rafts/caveolae microdomains, and/or (2) promote the release of intracellular soluble second messengers resulting in more widespread modifications of cells activity. The functional repercussions of the balance between these two signaling strategies regarding ADP-induced $[Ca^{2+}]_i$ oscillations, cell proliferation and type I collagen production by cardiac fibroblasts, were also investigated in this study.

CHAPTER 3. ORIGINAL RESEARCH PAPERS

The results obtained in this thesis were published or submitted for publication as original research papers, as follows:

Paper 1: **Certal M**, Vinhas A, Pinheiro AR, Ferreirinha F, Barros-Barbosa AR, Silva I, Costa MA, Correia-de-Sá P (2015). Calcium signaling and the novel anti-proliferative effect of the UTP-sensitive $P2Y_{11}$ receptor in rat cardiac myofibroblasts. Cell Calcium. 58: 518-533. DOI: 10.1016/j.ceca.2015.08.004; PMID: 26324417.

Paper 2: **Certal M**, Vinhas A, Barros-Barbosa A, Ferreirinha F, Costa MA, Correia-de-Sá P (2016). ADP-induced Ca^{2+} signaling and proliferation of rat ventricular myofibroblasts depend on phospholipase C-linked TRP channels activation within lipid rafts. Cell Signal (submitted for publication).

PAPER 1

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Calcium signaling and the novel anti-proliferative effect of the UTP-sensitive $P2Y_{11}$ receptor in rat cardiac myofibroblasts

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Abstract

During myocardial ischemia and reperfusion both purines and pyrimidines are released into the extracellular milieu, thus creating a signaling wave that propagates to neighboring cells via membrane-bound P2 purinoceptors activation. Cardiac fibroblasts (CF) are important players in heart remodeling, electrophysiological changes and hemodynamic alterations following myocardial infarction. Here, we investigated the role UTP on calcium signaling and proliferation of CF cultured from ventricles of adult rats.

Co-expression of discoidin domain receptor 2 and α-smooth muscle actin indicate that cultured CF are activated myofibroblasts. Intracellular calcium ($[Ca^{2+}]_i$) signals were monitored in cells loaded with Fluo-4 NW. CF proliferation was evaluated by the MTT assay.

UTP and the selective $P2Y_4$ agonist, MRS4062, caused a fast desensitizing $[Ca^{2+}]_i$ rise originated from thapsigargin-sensitive internal stores, which partially declined to a plateau providing the existence of Ca^{2+} in the extracellular fluid. The biphasic $[Ca^{2+}]_i$ response to UTP was attenuated respectively by $P2Y_4$ blockers, like reactive blue-2 and suramin, and by the $P2Y_{11}$ antagonist, NF340. UTP and the $P2Y_2$ receptor agonist MRS2768 increased, whereas the selective $P2Y_{11}$ agonist NF546 decreased, CF growth; MRS4062 was ineffective. Blockage of the $P2Y_{11}$ receptor or its coupling to adenylate cyclase boosted UTP-induced CF proliferation. Confocal microscopy and Western blot analysis confirmed the presence of $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors.

Data indicate that besides $P2Y_4$ and $P2Y_2$ receptors which are responsible for UTP-induced $[Ca^{2+}]_i$ transients and growth of CF, respectively, synchronous activation of the previously unrecognized $P2Y_{11}$ receptor may represent an important target for anti-fibrotic intervention in cardiac remodeling.

Introduction

Fibroblasts are highly abundant cells in the heart, where they contribute to myocardial architecture and function. Cardiac fibroblasts (CF) secrete growth factors, cytokines (such as IL-1β and TNF-α) and other signaling molecules in response to a variety of stimuli (e.g. mechanic, noxious, inflammatory); this is done in parallel to the well-known production of components of the extracellular matrix (ECM), such as collagen and fibronectin, and matrix metalloproteinases (Brown et al., 2005; Camelliti et al., 2005). Contraction of the collagen network by CF serves to direct mechanical force around cardiomyocytes, which can theoretically have a significant contribution to diastolic compliance. CF migration and proliferation, enhanced deposition of ECM and cardiomyocyte hypertrophy lead to cardiac remodeling and fibrosis (Brown et al., 2005) resulting in stiffness of the myocardium, which accompanies heart aging and numerous cardiac diseases, including ischemic and hypertensive heart failure and diabetic cardiomyopathy.

Due to their abundance, strategic localization and remarkable potential for activation, CF may serve as sentinel cells that sense myocardial injury and trigger inflammatory and reparative responses (Shinde et al., 2014). Disruption of cell-cell communication can lead to dysregulation of chemical, mechanical and electrical signals between cellular components of the heart. Involvement in myocardial dysfunction and arrhythmogenesis makes CF natural therapeutic targets in cardiac remodeling. Intense proliferative activity has been documented in CF infiltrating the infarcted heart; however, the molecular signals and intracellular pathways involved in CF proliferation remain poorly understood. Several growth factors (e.g. fibroblast growth factor-2, platelet-derived growth factor) released by a variety of cells (including macrophages, mast cells and cardiomyocytes) have been implicated in stimulation of the proliferative potential of fibroblasts.

During myocardial infarction there is a huge increase in the extracellular levels of adenine and uracil nucleotides (Wihlborg et al., 2006; Gerasimovskaya et al., 2002). This feature led to the hypothesis that various subtypes of metabotropic P2Y and ionotropic P2X purinoceptors play relevant roles in cardiac remodeling following an ischemic insult by targeting fibroblast cells activation and growth (Burnstock, 2006). However, in contrast to the compelling evidence for the extracellular signaling role of adenine nucleotides on cardiac fibroblasts (Lu et al.,

2012; Wakeno et al., 2006; McIntosh and Lasley, 2012; Chen et al., 2012), only recently attention has been given to the contribution of uracil nucleotides as autocrine/paracrine mediators in the heart (Braun et al., 2012; Yitzhaki et al., 2006).

The presence of UTP-sensitive $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors, as well as the UDP-sensitive $P2Y_6$ receptor, was demonstrated in neonatal rat cardiac myofibroblasts (Talasila et al., 2009), but no evidences have been produced regarding their role in these cells. Consensus exists in the literature suggesting that UTP transiently increases CF proliferation via the activation of $P2Y_2$ receptors, both in rats (Braun et al., 2010) and humans (Chen et al., 2012). This receptor subtype has also been involved on Ca^{2+} signaling in human valvular myofibroblasts (Liang et al., 2008). Despite these findings, some authors ascertain that UTP may be cardioprotective by decreasing infarct size and restoring cardiac function following myocardial infarction in rats (Yitzhaki et al., 2006). Recent studies suggest that $P2Y_{11}$-like receptors afford cardioprotection against ischemia and reperfusion (Djerada et al., 2013). As a matter of fact, loss-of-function polymorphisms of this receptor increase the risk of acute myocardial infarction in humans (Aminsten et al., 2007). The prominent action of the $P2Y_{11}$ receptor in controlling cardiomyocyte contractility and possible alterations in its function during cardiomyopathy suggests that this receptor may be a potential therapeutic target (Balogh et al., 2005), yet the role of this receptor on CF activation and growth has not been explored so far.

The $P2Y_{11}$ receptor has unique properties among all P2 receptor subtypes because it may dually couple to phospholipase C (PLC) and adenylate cyclase (AC) pathways, respectively via G_q and G_s proteins (Balogh et al., 2005; White et al., 2003). This makes the $P2Y_{11}$ receptor the only P2 receptor subtype capable of increasing intracellular cyclic AMP levels. Recruitment of distinct signaling cascades by the $P2Y_{11}$ receptor seems to be agonist specific, since while both ATP and UTP equally increase intracellular Ca^{2+} concentration the intracellular signaling pathway leading to Ca^{2+} recruitment diverge when the receptor is activated by each nucleotide (White et al., 2003).

In this context, this study was designed to investigate the effect of uracil nucleotides on $[Ca^{2+}]_i$ signaling and long-term growth of cultured CF from adult rat ventricles using recently available subtype-selective agonists and antagonists of

$P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors in order to find novel targets for therapeutic intervention in cardiac remodeling. We also attempted to investigate the intracellular signaling pathways implicated in $[Ca^{2+}]_i$ oscillations and proliferation of CF induced by UTP using specific enzymatic inhibitors of the two main pathways, PLC and AC.

Methods

Drugs and solutions

Adenosine 5′-triphosphate sodium salt (ATP), adenosine 5′-[γ-thio]triphosphate tetralithium salt (ATPγS), 2-aminoethoxydiphenylborane (2-APB), brefeldin A (BFA), caffeine, carbenoxolone (CBX), α-[2-(3-Chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-b-oxo-3-isoxazole propanenitrile (ESI-09), cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL 12,330a), 2-[(3,4-dihydro-2-phenyl-2H-1-benzopyran-6-yl)oxy]-5-nitro-pyridine (ORM-10103), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (thiazolyl blue formazan – MTT), direct red 80, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), fetal bovine serum (FBS), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), 1-methyl-3-isobutylxanthine (IBMX), mefloquine (MFQ), phosphate buffered saline system (PBS), picric acid, reactive blue-2 (RB-2), suramin sodium salt, uridine 5′-diphosphate (UDP), uridine 5′-triphosphate (UTP), type I collagenase, and cell culture reagents were from Sigma-Aldrich. (S)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride (H1152), 4,4'-(carbonylbis(imino-3,1-(4-methyl-phenylene)carbonylimino)) bis(naphthalene-2,6-disulfonic acid) tetrasodium salt (NF340), 4,4'-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)carbonylimino))-bis-1,3-xylene-alpha,alpha′-diphosphonic acid tetrasodium salt (NF546), N,N''-1,4-butanediylbis[N'-(3-isothiocyanatophenyl)thiourea (MRS2578), N^4-phenylpropoxycytidine-5'-O-triphosphate tetra(triethylammonium) salt (MRS4062), 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KB-R7943), thapsigargin, uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt (MRS2768),

^{10}Panx, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536) were obtained from Tocris Cookson Inc.. Brefeldin A, ESI-09, IBMX, H1152, mefloquine, U73122, SQ 22536, thapsigargin and KB-R7943 were dissolved in dimethylsulphoxide (DMSO). The other drugs were dissolved in distilled water. DMSO (<0.05% v/v) did not affect the parameters under study. All stock solutions were stored as frozen aliquots at -20ºC.

Fluo-4 NW dye was supplied by Molecular Probes (Invitrogen). ATP bioluminescence assay kit HS II was from Roche Applied Science. Bicinchoninic acid (BCA) Protein Assay Kit was from Pierce. Tissue culture plates: 96-well plates were purchased from Corning; FluoroDish plates for confocal microscopy were from World Precision Instruments; chamber slides were from Nunc. Polyvinyl difluoride (PVDF) membranes were obtained from Millipore.

Animals

Wistar rats (150-200 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21 ºC) and a regular light (06.30 –19.30 h) – dark (19.30–06.30 h) cycle, with food and water *ad libitum*. Animal handling and experiments were in accordance with the guidelines prepared by Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC).

Cell cultures

Rat cardiac fibroblasts were isolated from heart ventricles of adult Wistar rats by the explant technique in which fibroblasts migrate from minced tissue and grow in fibroblast growth medium (Song et al., 2012). Cells were cultured in DMEM medium supplemented with 15% fetal bovine serum (FBS), 1% of amphotericin B and 1% of penicillin/streptomycin, at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Medium was replaced twice a week. Primary cultures were maintained until near confluence (~3-4 weeks), then adherent cells were enzymatically released with 0.04% trypsin-EDTA solution plus 0.025% type I collagenase in phosphate-buffered saline (PBS). The resultant cell suspension was cultured and maintained in the same conditions mentioned above (Pinheiro et al., 2013a; Pinheiro et al., 2013b).

Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were measured with the calcium sensitive dye Fluo-4 NW (Molecular Probes, Invitrogen) in a multi detection microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments). In some of the experiments, single-cell $[Ca^{2+}]_i$ imaging was obtained using a laser scanning confocal microscope (FV1000, Olympus) in the time lapse mode (Pinheiro et al., 2013 a; Pinheiro et al., 2013b). Briefly, rat cardiac fibroblasts were seeded in flat bottom 96 well plates (Costar, Corning Inc.) at a density of $3x10^4$ cells/mL. Cells were cultured for 7 days in supplemented DMEM as described before. On the day of the experiment, cells were washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 11.2 mM glucose, pH 7.4) and incubated at 37ºC for 45 minutes with the cell-permeant fluorescent Ca^{2+} indicator, Fluo-4 NW (2.5 µM), in 1X HBSS, 20 mM HEPES and 2.5 mM probenecid. After removal of the fluorophore loading solution, cells were washed again twice and 150 µL of Tyrode's solution was added per culture well. For the recordings, temperature was maintained at 32ºC and readings were made with 5 seconds of interval, during approximately 30 minutes, using a tungsten halogen lamp. Fluorescence was excited at 485/20 nm and emission was measured at 528/20 nm. For single-cell $[Ca^{2+}]_i$ imaging, culture dishes were mounted on a thermostatic (32ºC) perfusion chamber on the stage of an inverted laser-scanning confocal microscope equipped with a 20x magnification objective lens (LUCPLFLN 20x PH; NA: 0.45). The chamber was continuously superfused (1 mL/min) with gassed (95% O_2, 5% CO_2, pH 7.4) Tyrode's solution and drugs were delivered using a multichannel valve controlled perfusion system. Changes in fluorescence of the Fluo-4 NW dye were detected in a time-lapse mode. Fluo-4 NW was excited with a 488 nm Multi-line Ar laser and the emitted fluorescence was detected at 510-560 nm, using the scanner of the confocal microscope. Time-lapse sequences were recorded at scanning rates with 20 seconds of interval for approximately 30 min, digitized, and processed off-line. Regions of interest were defined manually. For both methods, calcium measurements were calibrated to the maximal calcium load produced by the calcium ionophore, ionomycin (5 µM, 100% response) (Pinheiro et al., 2013a; Pinheiro et al., 2013b; Henriksen et al., 2006; Panupinthu et al., 2007).

Measurement of intracellular cyclic AMP

Rat cardiac fibroblasts were assayed for cyclic AMP production in the presence of a cyclic nucleotide phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, 100 μM final concentration in Tyrode's solution). Five min after IBMX application, incubation proceeded in the absence or in the presence of UTP (3 and 100 μM) for an additional 5-min period. The adenylate cyclase activator, forskolin (10 μM) was used as a positive control. Incubations were stopped by addition of 0.1% HCl. Cell lysates were harvested, neutralized and centrifuged. Supernatants were collected and acetylated according to the manufacturer's instructions. The samples were then analyzed for cyclic AMP content using a cyclic AMP-specific enzyme immunoassay (Cyclic AMP EIA kit, Cayman Chemicals Co.). Absorbance readings were done at 410 nm against blank. Cyclic AMP concentration in each sample was expressed as pmol per mg of protein.

Extracellular ATP quantification by bioluminescence

Extracellular ATP was quantified by the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science) using a multi detection microplate reader (Synergy HT, BioTek Instruments), as previously described by our group (Pinheiro et al., 2013a; Pinheiro et al., 2013b). Briefly, cells were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL for 7 days. At the beginning of the experiment, cells were washed twice with Tyrode's solution at 37ºC and let rest during 30 minutes (basal), after which samples were collected (100 μL). MRS 4062 (10 μM) contacted with the cells for 5 min either in the absence or in the presence of release modifiers, which were added to the incubation fluid at least 5 min before the $P2Y_4$ agonist. Collected samples were immediately snap-frozen and stored at -80ºC. Luciferin-luciferase experiments were performed at room temperature and light emission acquisition was performed 20 seconds after addition of luciferin-luciferase to the collected sample.

Cell viability/proliferation

Viability/proliferation studies included the MTT assay as previously described (Costa et al., 2011; Pinheiro et al., 2013a; Pinheiro et al., 2013b). Rat cardiac fibroblasts were seeded in flat bottom 96 well plates at a density of $3x10^4$

cells/mL and cultured in supplemented DMEM as described before. Cell cultures were routinely monitored by phase contrast microscopy and characterized at days 1, 7, 14, 21 and 28. The MTT assay consists on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan reaction product by viable cells. In the last 4 h of each test period, cells were incubated with 0.5 mg/mL of MTT for 4 h in the conditions referred above. The medium was carefully removed, decanted and the stained product dissolved with DMSO before absorbance (A) determination at 600 nm using a microplate reader spectrometer (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments). Results were expressed as A/well.

Type I collagen determination

Type I collagen determination was performed using the Sirius Red staining assay. Rat cardiac fibroblasts were cultured as described for the viability/proliferation studies. The staining protocol was adapted from Tullberg-Reinert & Jundt (1999) (Tullberg-Reinert et al., 1999). Cell layers were washed twice in PBS before fixation with Bouin's fluid for 1 h. The fixation fluid was removed by suction and the culture plates were washed by immersion in running tap water for 15 minutes. Culture dishes were allowed to air dry before adding the Sirius Red dye (Direct Red 80). Cells were stained for 1 h under mild shaking on a microplate shaker. To remove non-bound dye, stained cells were washed with 0.01 N hydrochloric acid and then dissolved in 0.1 N sodium hydroxide for 30 minutes at room temperature using a microplate shaker. Optical density was measured at 550 nm using 0.1 N sodium hydroxide as blank (Tullberg-Reinert et al., 1999; Pinheiro et al., 2013a; Pinheiro et al., 2013b). Results were expressed as A/well.

Immunocytochemistry

Rat cardiac fibroblasts were seeded in chamber slides at a density of $2.5x10^4$ cells/mL and allowed to grow for 7-28 days. Cultured cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes, washed 3 times in PBS (10 minutes each) and, subsequently, incubated with blocking buffer I (10% FBS, 1% bovine serum albumin (BSA), 0.1% Triton X, 0.05% NaN_3 in PBS) for 1 h. Primary

antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN_3), were applied [mouse anti-porcine vimentin 1:250 (DAKO); goat anti-human DDR2 1:25 (Santa Cruz); mouse anti human α-smooth muscle actin-FITC 1:250 (Sigma); rabbit anti-rat $P2Y_2$ 1:100 (Alomone), rabbit anti-rat $P2Y_4$ 1:75 (Alomone), rabbit anti-human $P2Y_{11}$ 1:50 (Alomone), rabbit anti-human connexin 43 1:600 (Abcam)] and the slides incubated overnight at 4ºC. After incubation, cells were washed 3 times in PBS (10 min each). The donkey anti-rabbit IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 488 and donkey anti-goat IgG Alexa Fluor 633 secondary antibodies (Invitrogen) were diluted in blocking buffer II and applied for 1h at dark. A last wash was performed with PBS and glass slides were mounted with VectaShield medium and stored at 4ºC. Observations were performed and analysed with a laser scanning confocal microscope (FV1000, Olympus) (Alqallaf et al., 2009; Noronha-Matos et al., 2012; Pinheiro et al., 2013a; Pinheiro et al., 2013b)

SDS–PAGE and Western blotting

Cardiac fibroblasts were homogenized in a lysis buffer with the following composition: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS and a protease inhibitor cocktail. Protein content of the samples was evaluated using the BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Samples were solubilized in SDS reducing buffer (0.125 mM Tris-HCl, 4% SDS, 0.004% bromphenol blue, 20% glycerol, and 10% 2-mercaptoethanol, pH 6.8 at 70ºC for 10 minutes), subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (MilliPore, MA). Protein loads were 15 µg for connexin 43 (Cx43) and 100 µg for $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors. The membranes were blocked for 1 h in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 + 5% BSA. Membranes were subsequently incubated with rabbit anti-rat $P2Y_2$ 1:200 (Alomone), rabbit anti-rat $P2Y_4$ 1:300 (Alomone), rabbit anti-human $P2Y_{11}$ 1:200 (Alomone) and rabbit anti-human Cx43 1:6000 (Abcam) in the above blocking buffer overnight at 4ºC. Membranes were washed three times for 10 minutes in 0.1% Tween 20 in TBS and then incubated with donkey anti-rabbit IgG (HRP) 1:30000 (Abcam) secondary antibody, for 60 minutes at room temperature. For comparison purpose, the

membranes were also incubated with rabbit anti-human β-tubulin 1:2500 (Abcam) or rabbit anti-human β-actin 1:2500 (Abcam) antibodies following the procedures described above. Membranes were washed three times for 10 minutes and antigen-antibody complexes were visualized by chemiluminescence with an ECL reagent using the ChemiDoc MP imaging system (Bio-Rad Laboratories).

Presentation of data and statistical analysis

Data are expressed as mean ± S.E.M. from an *n* number of individuals. Data from different individuals performed in triplicate were evaluated by one-way analysis of variance (ANOVA). Statistical differences found between control and drug-treated cultures were determined by the Bonferroni's method. *P* values < 0.05 were considered to represent significant differences.

Results

Characterization of adult rat cardiac fibroblasts in culture

The fibroblast nature of cells in culture was confirmed by immunocytochemistry. Adult rat CF exhibited positive immunoreactivity against discoidin domain receptor 2 (DDR2) (Figure 4B and 4E), a collagen specific receptor tyrosine kinase that is the most specific marker for CF and myofibroblasts (Morales et al., 2005; Goldsmith et al., 2010). Cells also stained positive for vimentin (Figure 4A and 4C), the intermediate protein filament considered a reliable fibroblast-cell marker (Agocha and Eghbali-Webb, 1997), and α-smooth muscle actin (α–SMA) (Figure 4D and 4F), a myofilament protein that typically is expressed by activated fibroblasts (myofibroblasts) (Brown et al., 2005). This staining pattern indicates that adult rat CF used in this study present a myofibroblast phenotype, which is the CF lineage most committed to myocardial remodeling (Stephan, 2011). Rapid and spontaneous transition to myofibroblast phenotype is characteristic for CF cultured in standard two-dimensional conditions (Rohr, 2011).

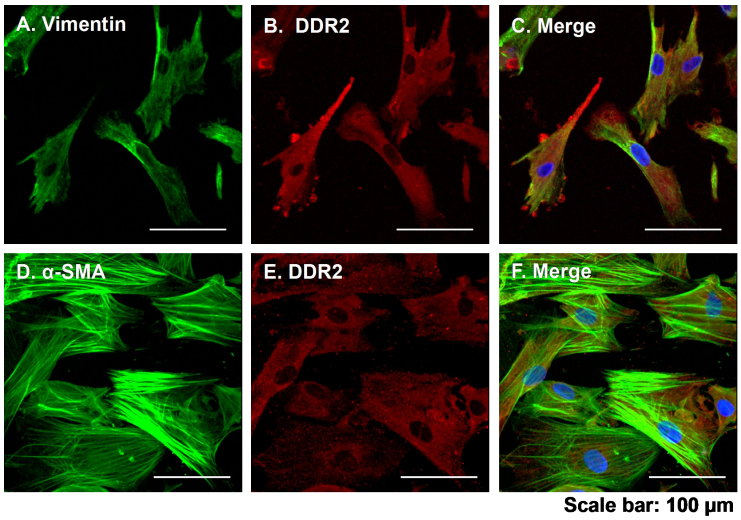

Figure 4 - Immunocytochemical staining of CF isolated from heart ventricles of adult rats. Cells exhibited positive immunoreactivity against vimentin, which has been described as a reliable fibroblast marker (green, A), α-smooth muscle actin (α-SMA), a myofilament protein that typically is expressed by activated fibroblasts (myofibroblasts) (green, D) and discoidin domain receptor 2 (DDR2), a collagen specific receptor tyrosine kinase that is the most specific marker for cardiac fibroblasts and myofibroblasts (red, B and E). Panels C and F show merge of vimentin and DDR2, and α-SMA and DDR2, respectively. Yellow denotes co-localization. Scale bar: 100 µm.

UTP stimulates fast Ca^{2+} recruitment from intracellular stores, which is kept at high levels via Ca^{2+} influx from the extracellular space

UTP (0.03-300 µM) concentration-dependently increased $[Ca^{2+}]_i$ above the control level (Tyrode's solution) when applied to 7-day cultures of adult rat CF (Figure 5A). UTP typically produced a biphasic response (Figure 5B). UTP (3 µM, n=26) caused a fast (within seconds) rise in $[Ca^{2+}]_i$ (phase I), which attained 33±1% of the maximal calcium load produced by ionomycin (5 µM, 100% response); beyond this point, $[Ca^{2+}]_i$ levels declined to a plateau of elevated $[Ca^{2+}]_i$ (phase II) that remained fairly constant. The initial $[Ca^{2+}]_i$ rise (phase I) produced by UTP decreased ($p<0.05$) in the presence of the selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (5 µM) (Thastrup et al., 1990), which is known to accelerate the depletion of intracellular Ca^{2+} stores when applied together with internal Ca^{2+} mobilizing agents, such as caffeine (10 mM, n=5) (Figure 5C). Removal of external Ca^{2+} (plus EGTA, 100 µM, n=6) significantly ($p<0.05$) depressed the late component of UTP (3 µM, n=6) response (phase II), keeping unaltered the initial fast rise (Figure 5D). Ca^{2+} influx through the Na^+/Ca^{2+} exchanger (NCX) operating in the reverse mode does not account for UTP-induced $[Ca^{2+}]_i$ rise in rat CF because selective inhibition of NCX with anti-

arrhythmogenic compounds, like KB-R7943 (10 μM, n=5) (Ladilov et al., 1999) or ORM-10103 (3 μM, n=4) (Jost et al., 2013), were devoid of effect (data not shown).

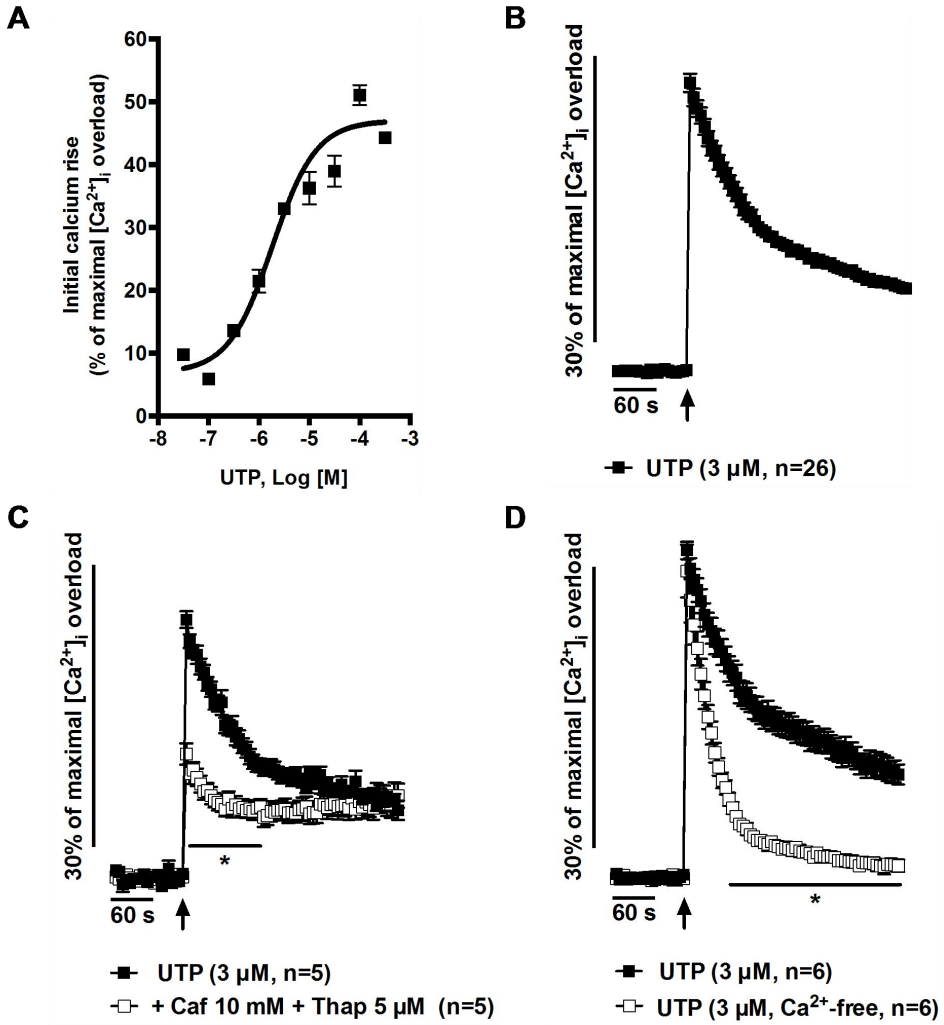

Figure 5 - UTP stimulates fast Ca^{2+} recruitment from intracellular stores in cultured CF, which is kept at high levels via Ca^{2+} influx from the extracellular space. Panel A depicts the concentration-response curve of $[Ca^{2+}]_i$ rises produced by UTP (0.03-300 μM). Panels B, C and D illustrate UTP (3 μM)-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients in cultured CF from adult rats loaded with the fluorescent calcium indicator, Fluo-4 NW (2.5 μM, see Methods) in control conditions (B) and after depletion of intracellular Ca^{2+} stores with thapsigargin (5 μM) plus caffeine (10 mM) (C) or after removal of extracellular Ca^{2+} (Ca^{2+}-free medium plus EGTA, 100 μM, D). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). Black arrows indicate the time of UTP (3 μM) application. Each point represents pooled data from an n number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from UTP (3 μM) alone.

Pharmacological characterization of P2 receptors mediating UTP-induced $[Ca^{2+}]_i$ oscillations in adult rat cardiac fibroblasts

Regarding the initial $[Ca^{2+}]_i$ rise, UTP (100 μM, n=11) was about equipotent to ATP (100 μM, n=16) and ATγS (100 μM, n=4, a stable ATP analogue) (Figure 6A), but it was much potent (p<0.05) than UDP (100 μM, n=4), which may be

originated from the extracellular hydrolysis of UTP by ectonucleotidases (Figure 6B). Involvement of the UDP-sensitive $P2Y_6$ receptor was further ruled out since blockade of this receptor with a selective antagonist, MRS2578 (300 nM) (Mamedova et al., 2004), did not affect UTP- and UDP-induced $[Ca^{2+}]_i$ oscillations (Figure 6B). The effect of UTP (3 μM) on $[Ca^{2+}]_i$ oscillations was attenuated ($p<0.05$) by suramin (100 μM, n=7) and reactive blue-2 (RB-2, 100 μM, n=7) (Figure 7Ai and 7Aii); two broad spectrum P2 receptor antagonists which on their own were devoid of effect on $[Ca^{2+}]_i$ oscillations (data not shown).

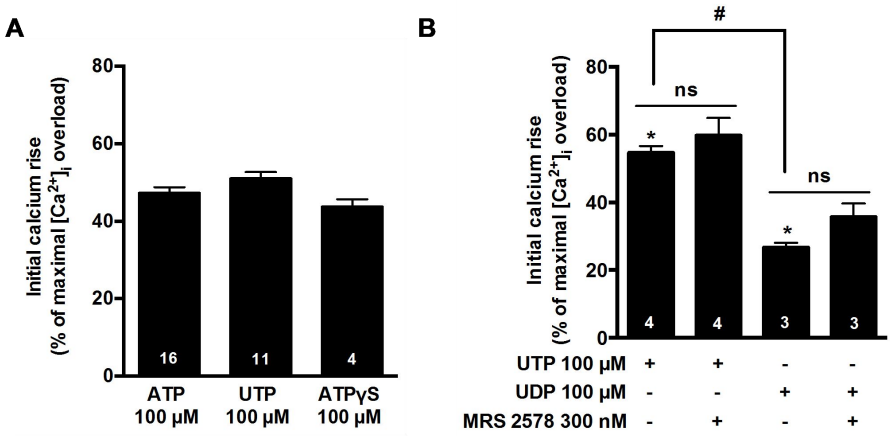

Figure 6 - UTP-induced $[Ca^{2+}]_i$ transients in cultured CF of adult rats are mediated by a P2Y purinoceptor that is equally sensitive to ATP and ATPγS, but not to UDP. Panel A represents the magnitude of the initial $[Ca^{2+}]_i$ response of ATP (100 μM), UTP (100 μM) and ATPγS (100 μM). Panel B compares the magnitude of $[Ca^{2+}]_i$ peak produced by UTP (100 μM) and UDP (100 μM) applied in the absence and in the presence of the selective $P2Y_6$ receptor antagonist, MRS 2578 (300 nM). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). Each bar represents pooled data from an n number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *$p<0.05$ represent significant differences from control values obtained in the absence of test drugs, #$p<0.05$ represent significant differences from UTP (100 μM) alone. Not significant: ns.

Pharmacological data suggest the participation of UTP-sensitive P2 purinoceptors operating $[Ca^{2+}]_i$ oscillations, which can be of $P2Y_2$, $P2Y_4$ or $P2Y_{11}$ subtypes as all these receptors were identified in adult rat CF by immunofluorescence confocal microscopy and Western blot analysis (Figure 7B). Our data also show that the selective $P2Y_4$ receptor agonist, MRS 4062 (10 μM, n=7) (Maruoka et al., 2011), mimicked the effect of UTP on $[Ca^{2+}]_i$ oscillations, whereas selective activation of $P2Y_2$ and $P2Y_{11}$ receptors with MRS 2768 (10 μM, n=5) (Ko et al., 2008) and NF546 (10 μM, n=5) (Meis et al., 2010), respectively, caused only minor effects typically lacking the initial fast $[Ca^{2+}]_i$ rise (Figure 7C and 7D). The $P2Y_4$ receptor-induced fast $[Ca^{2+}]_i$ rise desensitizes rapidly with repeated and/or prolonged applications of UTP (30 μM) or MRS 4062 (10 μM) (Figure 7E).

To investigate this, we performed single-cell $[Ca^{2+}]_i$ imaging experiments using a laser scanning confocal microscope in the time-lapse mode; agonists were applied to the superfusion fluid during 1-min followed by 8-min washout intervals between consecutive applications.

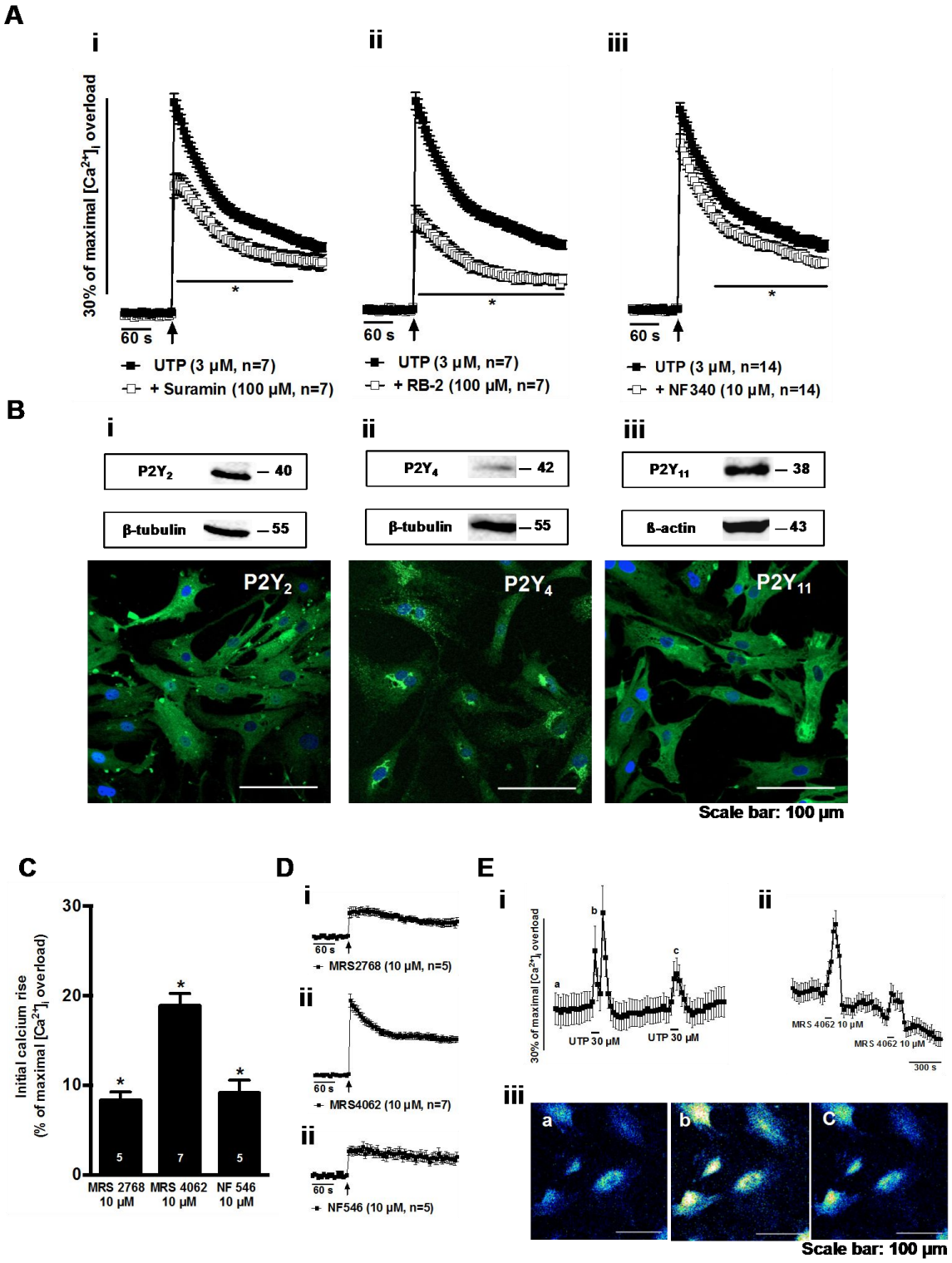

Figure 7 - UTP-induced $[Ca^{2+}]_i$ rises depend on a dominant $P2Y_4$ receptor activation in rat CF that also co-express $P2Y_2$ and $P2Y_{11}$ receptor subtypes. Panel A shows the amplitude of UTP (3 µM)-

induced $[Ca^{2+}]_i$ rise in the absence and in the presence of two non-selective P2 receptors antagonists, suramin (100 μM, Ai) and reactive blue-2 (RB-2, 100 μM, Aii), and of the selective $P2Y_{11}$ receptor antagonist, NF 340 (10 μM, Aiii). Each point represents pooled data from an n number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from UTP (3 μM) alone. Panel B shows immunofluorescence confocal micrographs and representative immunoblots of $P2Y_2$ (Bi), $P2Y_4$ (Bii) and $P2Y_{11}$ (Biii) receptors. (-actin and (-tubulin were used as references. Images are representative of three independent experiments; scale bar is 100 μm. Panels C and D compare the magnitude of $[Ca^{2+}]_i$ transients produced by selective agonists of $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors, respectively MRS 2768 (10 μM), MRS 4062 (10 μM) and NF 546 10 μM), on rat CF maintained for 7 days in culture. Black arrows indicate the time of drugs application. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). Each bar/point represents pooled data from an n number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from control values obtained in the absence of test drugs. Panel E represents $[Ca^{2+}]_i$ oscillations induced by UTP (3 μM, Ei) and MRS 4062 (10 μM, Eii) applied to the superfusion fluid during 1-min followed by 8-min washout intervals between consecutive applications. Changes in fluorescence were detected in the time-lapse mode using a laser scanning confocal microscope. Horizontal lines indicate the time of drugs application. Fluorescence confocal micrographs were obtained at the indicated time points (a, b and c) (Eiii). Scale bar is 100 μm.

Interestingly, the plateau phase, but not the initial $[Ca^{2+}]_i$ rise, induced by UTP (3 μM) was partially decreased ($p<0.05$) by blocking the $P2Y_{11}$ receptor with NF340 (10 μM, n=14) (Meis et al., 2010) (Figure 7Aiii). As previously mentioned, the $P2Y_{11}$ receptor may positively couple to both PLC and AC, whereas other UTP-sensitive receptors, namely $P2Y_2$ and $P2Y_4$, lead to accumulation of inositol 1,4,5-trisphosphate (IP_3) and subsequent mobilization of Ca^{2+} from internal stores through the activation of PLC (Kugelgen, 2006). Inhibition of either PLC with U73122 (30 μM, n=5) or the IP_3-receptor with 2-APB (50 μM, n=6) decreased ($p<0.05$) both the initial rise (phase I) and the plateau (phase II) of UTP (3 μM)-induced $[Ca^{2+}]_i$ response (Figure 8Ai, 8Aii and 8D). Participation of a $P2Y_{11}$ receptor-coupled AC pathway in the plateau phase of $[Ca^{2+}]_i$ response is supported by our findings showing that inhibition of AC either with MDL12,330a (10 μM, n=9) or with SQ 22536 (30 μM, n=4) mimicked the blocking effect of the $P2Y_{11}$ receptor antagonist, NF340 (10 μM, n=14) (Figure 8Aiii and 8Aiv, for comparison see Figure 7Aiii). Figure 8Dii shows that the inhibitory effects of U73122 (30 μM) and MDL12,330a (10 μM) were additive on UTP (3 μM)-induced $[Ca^{2+}]_i$ plateau (phase II), but the same was not verified regarding the initial $[Ca^{2+}]_i$ rise (phase I) (Figure 8Di). These findings suggest that PLC and AC act independently in a cooperative manner to keep $[Ca^{2+}]_i$ at high levels during the plateau (phase II) of the UTP response, while the PLC/IP_3 is the dominant pathway responsible for the initial $[Ca^{2+}]_i$ rise.

UTP increases intracellular cyclic AMP accumulation leading to activation of an EPAC-mediated signaling pathway in cardiac fibroblasts of adult rats

Figure 8B shows that UTP (3 and 100 μM) concentration-dependently increased intracellular cyclic AMP levels, strengthening our assumption that the $P2Y_{11}$ receptor is involved in UTP signaling in adult rat CF considering that this is the only P2 subtype operating increases in intracellular cyclic AMP. Most cyclic AMP effects are attributed to the activation of protein kinase A (PKA), yet several cyclic AMP-mediated cellular events may be insensitive to PKA inhibition. Figure 8Ci shows that UTP (3 μM)-induced $[Ca^{2+}]_i$ rise was not modified in the presence of the potent PKA inhibitor, H-89 (10 μM, n=5), regardless of the inhibitory effects obtained by blocking the activity of AC with MDL12,330a (10 μM) or SQ 22536 (30 μM) (Figure 8A). This prompted us to investigate novel cyclic AMP targets, namely the exchange protein directly activated by cyclic AMP (EPAC). Selective inhibition of EPAC with ESI-09 (10 μM, n=5) significantly (p<0.05) decreased UTP (3 μM)-induced $[Ca^{2+}]_i$ rise in adult rat CF (Figure 8Cii), suggesting that this pathway mediates UTP actions downstream activation of membrane-bound $P2Y_{11}$ receptors.

UTP-induced $[Ca^{2+}]_i$ accumulation partially depends on ATP release from rat cardiac fibroblasts via hemichannels containing connexin-43

Stimulated fibroblasts can release substantial amounts of ATP leading to supplementary activation of membrane-bound P2 purinoceptors (see e.g. Pinheiro et al., 2013 a and b). Such a mechanism may contribute to sustain elevated $[Ca^{2+}]_i$ levels during the late plateau (phase II) response to UTP. Using the luciferin-luciferase bioluminescence assay, we show here that selective activation of the $P2Y_4$ receptor with MRS 4062 (10 μM, n=6) significantly (p<0.05) increased ATP release from CF isolated from adult rat ventricles compared to the control situation where the cells were exposed to Tyrode's solution (Figure 9A). Unfortunately, contrary to MRS 4062 (10 μM), UTP interferes with ATP determinations using the luciferin-luciferase bioluminescence assay given rise to false positive results, which precluded further tests with this compound.

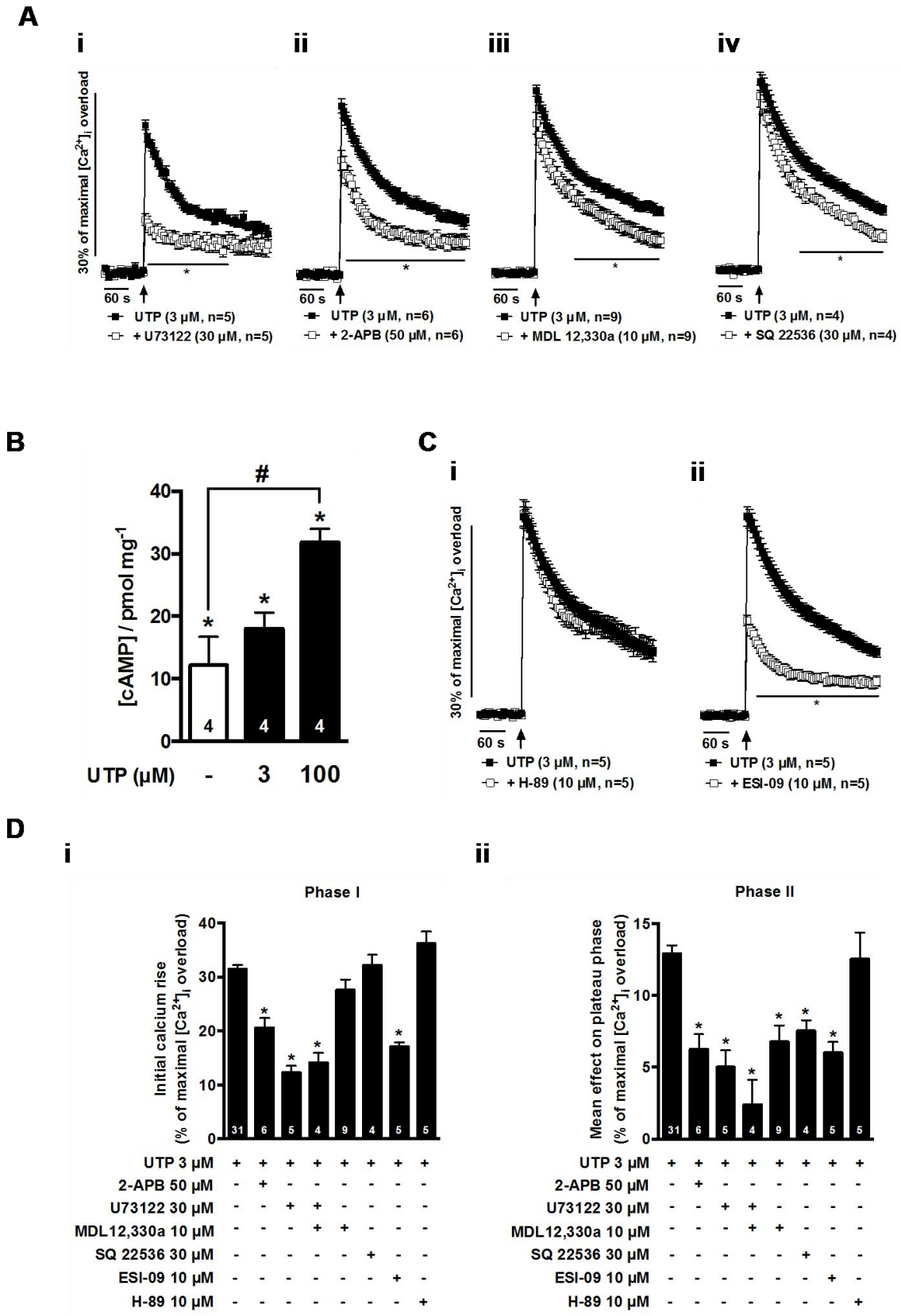

Figure 8 - Role of phospholipase C (PLC) and adenylate cyclase (AC) pathways on UTP-induced $[Ca^{2+}]_i$ transients in CF of adult rats. Panel A represents $[Ca^{2+}]_i$ oscillations induced by UTP (3 µM) in the absence and in the presence of PLC, IP_3-receptor and AC inhibitors, respectively U73122 (30 µM, Ai), 2-APB (50 µM, Aii), MDL12,330a (10 µM, Aiii) and SQ 22536 (30 µM, Aiv). Black arrows indicate the time of UTP (3 µM) application; inhibitors were applied at least 6 min before

UTP. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). The vertical bars represent S.E.M.. *p<0.05 represent significant differences from UTP (3 μM) alone. Panel B shows UTP-induced cAMP accumulation in rat cardiac fibroblasts. Each bar represents pooled data from an n number of experiments. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from baseline values; #p<0.05 represent significant differences from control values obtained in the absence of the nucleotide. Panel C represents $[Ca^{2+}]_i$ oscillations induced by UTP (3 μM) in the absence and in the presence of PKA and EPAC inhibitors, respectively H-89 (10 μM, Ci) and ESI-09 (10 μM, Cii). The vertical bars represent S.E.M.. *p<0.05 represent significant differences from UTP (3 μM) alone. Panel D compares the influence of these inhibitors on the initial $[Ca^{2+}]_i$ rise (phase I, 0-15 s, Di) and delayed plateau (phase II, 150-300 s, Dii) response produced by UTP (3 μM). Each bar represents pooled data from an n number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from UTP (3 μM) alone.

Hemichannels containing connexins and pannexins are putative mediators of ATP translocation into the extracellular milieu in fibroblasts (Pinheiro et al., 2013a; Pinheiro et al., 2013b). It has been shown that connexin-43 (Cx43) plays an important role in the differentiation of CF to activated myofibroblasts (Asazuma-Nakamura et al., 2009). Using immunofluorescence confocal microscopy and Western blot analysis, we confirmed that CFs from adult rats express Cx43 (Figure 9B). Carbenoxolone (CBX, 300 μM), the most potent inhibitor of Cx43-containing hemichannels significantly ($p<0.05$) attenuated ATP release triggered by MRS 4062 (10 μM, n=7) (Figure 9A), as well as the sustained $[Ca^{2+}]_i$ accumulation following UTP (3 μM, n=5) application (Figure 9C and 9D). Compounds that affect hemichannels pore permeability, like the Rho kinase inhibitor, H1152 (3 μM, n=4) (Sasaki et al., 2002), also decreased ($p<0.05$) the release of ATP induced by the $P2Y_4$ receptor agonist, MRS 4062 (10 μM) (Figure 9A). A similar interference was observed with the IP_3-receptor inhibitor, 2-APB (50 μM, n=4) (Figure 9A). The inhibitory effect of CBX (300 μM) on the late component (phase II) of UTP (3 μM)-induced $[Ca^{2+}]_i$ response was not reproduced by the selective pannexin-1 (Panx1) mimetic inhibitory peptide, ^{10}Panx (100 μM, n=5) (Wang et al., 2007) (Figure 9D), neither by mefloquine (MFQ, 3 μM, n=4), which is known to completely block Cx36- and Cx50-containing hemichannels (Cruikshank et al., 2004).

Involvement of ATP release by vesicle trafficking and exocytotic granule secretion was assessed using the vesicular transport inhibitor, brefeldin A (BFA) (Nebenfuhr et al., 2002). No statistical significant ($p<0.05$) differences were found in UTP (3 μM)-induced $[Ca^{2+}]_i$ responses obtained in the absence and in the presence of BFA (20 μM, n=8, Figure 9D).

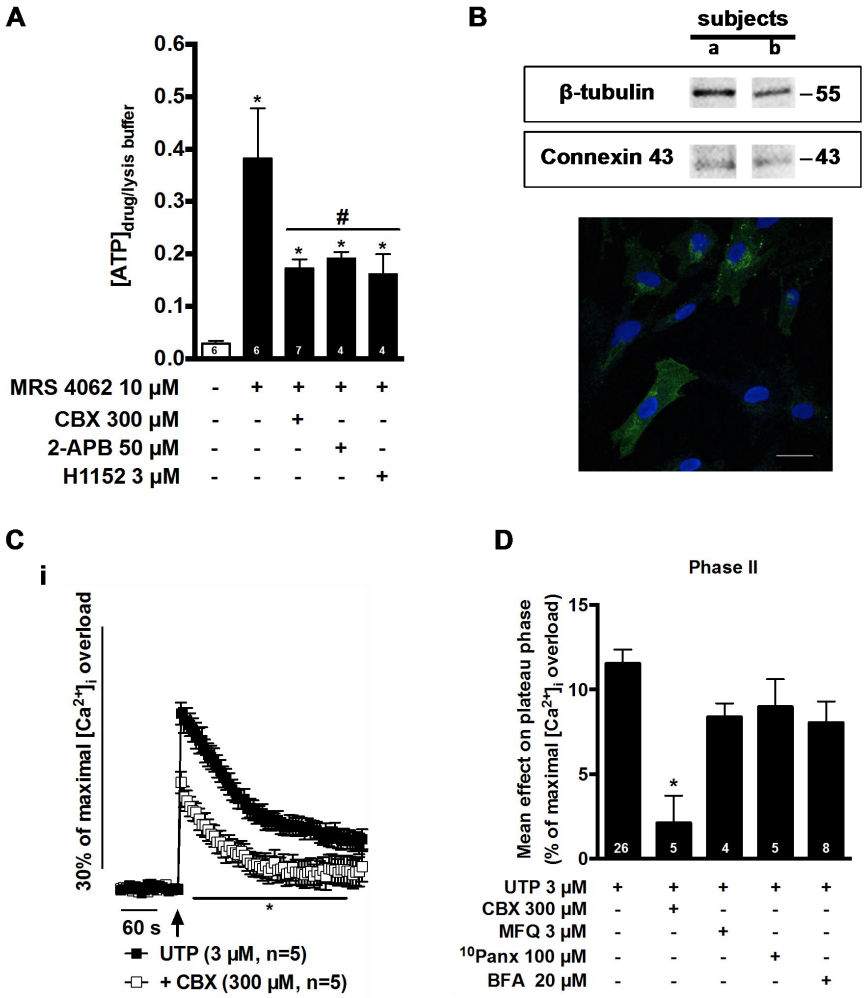

Figure 9 - UTP-induced $[Ca^{2+}]_i$ accumulation partially depends on ATP release from rat cardiac fibroblasts via hemichannels containing connexin-43 (Cx43). Panel **A** represents the ATP content of samples collected from rat cardiac fibroblast culture media 5 min after incubation with MRS 4062 (10 µM) in the absence and in the presence of carbenoxolone (300 µM), 2-APB (50 µM) and H1152 (3 µM). Each bar represents pooled data from an n number of experiments. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from control values obtained when only Tyrode's solution was added to the cultures; #p<0.05 represent significant differences from the effect of MRS 4062 (10 µM) alone. Panel **B** shows the presence of Cx43 immunoreactivity in cultured CF; shown is a confocal micrograph and representative immunoblots from two different individuals (a and b). Images are representative of three independent experiments; scale bar is 100 µm. Panel **C** represents $[Ca^{2+}]_i$ oscillations induced by UTP (3 µM) in the absence and in the presence of the Cx43-containing hemichannel blocker, carbenoxolone (CBX, 300 µM). Panel **D** shows the influence of CBX (300 µM), Mefloquine (MFQ, 3 µM), ^{10}Panx (100 µM), and the vesicular transport inhibitor, brefeldin A (20 µM), on UTP (3 µM)-induced $[Ca^{2+}]_i$ plateau (phase II, 150-300 s). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Each point/bar represents pooled data from an n number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from UTP (3 µM) alone.

$P2Y_2$ receptor-induced cardiac fibroblasts growth is partially counteracted by co-activation of $P2Y_{11}$ receptors

Previous reports from the literature suggest that UTP promotes growth of CF through the activation of the $P2Y_2$ receptor (Braun et al., 2010), but there are

reasons to believe that profibrotic responses mediated by this receptor are transient (<24 h) and the interplay with other receptors sensitive to UTP may also play a role. Figure 10Ai shows that CF cultures from adult rats grown for 28 days in control conditions proliferate slowly throughout the test period (Lindner et al., 2012). Results concerning type I collagen production followed a similar pattern to that obtained in the MTT (viability/proliferation) assay (Figure 10Aii), indicating that under the present experimental conditions the amount of extracellular matrix being produced depend directly on the number of viable cells in the culture.

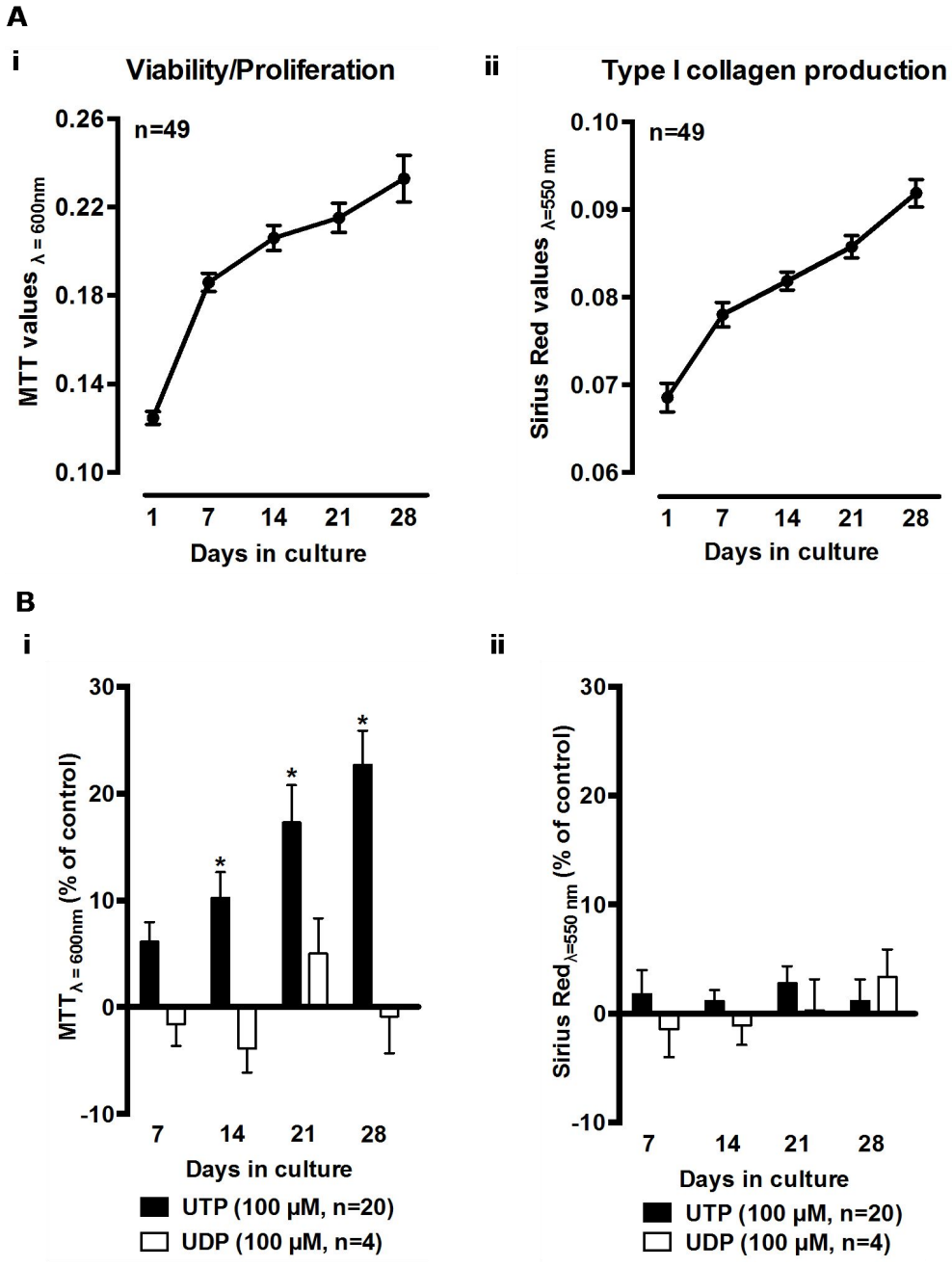

Figure 10 - Role of uracil nucleotides, UTP and UDP, on proliferation/viability and type I collagen production by adult rat CF grown for 28 days in culture. **Panel A** represents viability/proliferation of

cells measured by the MTT assay (Ai) and total type I collagen production assessed by Sirius Red staining (Aii); the ordinates represent absorbance determinations at 600 nm and 550 nm per well at certain time points, respectively for the MTT assay and the Sirius Red staining. In **panel B** the ordinates represent UTP (100 μM)- or UDP (100 μM)-induced changes in cell growth (MTT values, Bi) and type I collagen production (Sirius Red values, Bii) as compared to controls in the absence of uracil nucleotides at the same points (see panels Ai and Aii). Zero represents similarity between the two values (uracil nucleotide vs control); positive and negative values represent facilitation or inhibition of either cell growth or type I collagen production relative to control data obtained at the same time points. Each bar represents pooled data from an n number of individuals; four replicas were performed for each individual experiment. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from control values obtained in the absence of test drugs, #p<0.05 represent significant differences from UTP (100 μM) alone.

Incubation of CF cultures with UTP (100 μM, n=20), but not with UDP (100 μM, n=4), increased (p<0.05) CF proliferation from the first week onwards. At days 21 and 28, the MTT values increased (p<0.05) by 17±3% and 22±3%, respectively, in the presence of UTP (100 μM, n=20) as compared to control values (Figure 10Bi). UTP and UDP (100 μM) failed to increase (p>0.05) type I collagen production above the control level indicating that uracil nucleotides act predominantly to promote proliferation/viability of adult rat CF (Figure 10Bii and Table 5).

Table 5 - Normalization of type I collagen production values to cell growth (MTT assay values) in CF from adult rats grown in culture during 28 days. UTP (100 μM) was incorporated in culture media, and, therefore, contacted with the cells throughout the whole assay. Because the amount of type I collagen produced depends on the number of viable cells in culture, we normalized Sirius Red absorbance values by the corresponding MTT values. Values are means ± SEM from an *n* number of individuals; four replicas were performed for each individual experiment. *p<0.05 represent significant differences from control values obtained in the absence of UTP (100 μM).

Type I collagen (λ=550 nm) / MTT (λ= 600 nm)		
Days	**Control (n=44)**	**UTP 100 μM (n=17)**
7	0.476 ± 0.025	0.453 ± 0.027
14	0.484 ± 0.018	0.426 ± 0.018
21	0.523 ± 0.025	0.407 ± 0.018 *
28	0.540 ± 0.026	0.377 ± 0.019 *

In order to characterize the P2Y receptor subtype involved on UTP-induced proliferation, CF cultures were treated during 28 days with enzymatically-stable and selective $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptor agonists. The $P2Y_2$ receptor agonist, MRS 2768 (30 μM, n=4), but not the $P2Y_4$ agonist, MRS 4062 (10 μM, n=4), increased (p<0.05) CF proliferation (Figure 11A and 11B). Conversely, selective activation of the $P2Y_{11}$ receptor with NF 546 (10 μM, n=9) significantly (p<0.05) decreased CF growth (Fig. 11C). None of these agonists affected significantly

(p>0.05) type I collagen production with the exception of a small inhibitory effect verified at day 28 in cultures treated with MRS 4062 (10 μM) (Figure 11B).

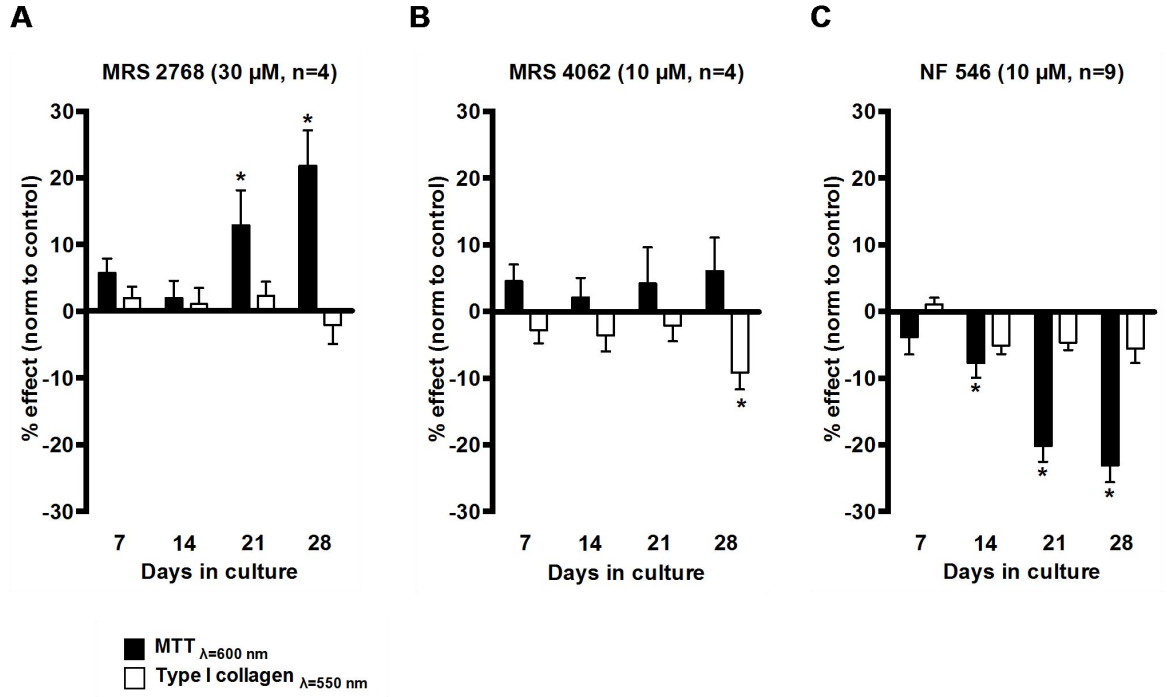

Figure 11 - Effects of selective $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptor agonists on proliferation/viability and type I collagen production by adult rat CF grown for 28 days in culture. MRS 2768 (30 μM, $P2Y_2$ agonist), MRS 4062 (10 μM, $P2Y_4$ agonist) or NF 546 (10 μM, $P2Y_{11}$ agonist) were incorporated in culture media, and, therefore, contacted with the cells during the whole assay. The ordinates represent changes in cell growth (MTT values) and type I collagen production (Sirius Red values) as compared to controls in the absence of test drugs at the same points (see Fig. 6). Zero represents similarity between the two values (drugs vs control); positive and negative values represent facilitation or inhibition of either cell growth or type I collagen production relative to control data obtained at the same time points. Each bar represents pooled data from four individuals; four replicas were performed for each individual experiment. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from control values obtained in the absence of test drugs.

Involvement of the $P2Y_2$ receptor on CF proliferation was further confirmed by our results showing that suramin (Figure 12Ai), but not reactive blue-2 (Figure 12Aii), attenuated (p<0.05) UTP (100 μM)-induced CF growth, when the two antagonists were applied in a concentration (30 μM) that, on its own, was devoid of effect on cell proliferation. UTP (100 μM)-induced CF growth increased further (p<0.05) upon blocking the $P2Y_{11}$ receptor activation with NF 340 (10 μM, n=6) (Figure 12Aiii); this finding reiterates the anti-proliferative action of the $P2Y_{11}$ receptor on CF growth. Interestingly, blockade of Cx43-containing hemichannels with CBX (10 μM) and, thereby, ATP release from the cells also boosted CF proliferation induced by UTP (100 μM) in a similar manner to that obtained in the presence of the $P2Y_{11}$ receptor antagonist, NF 340 (10 μM) (Figure 12B).

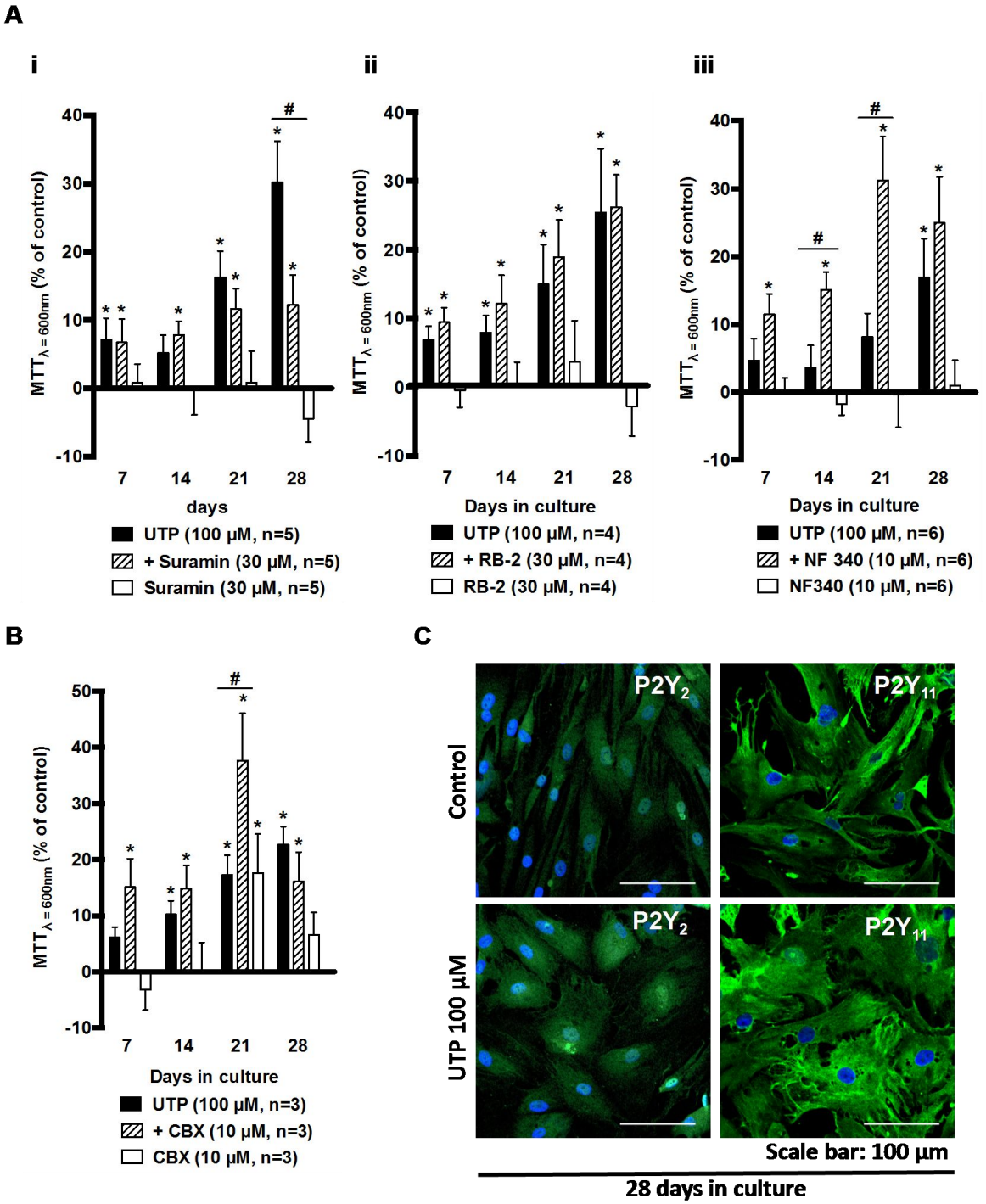

Figure 12 - The proliferative action of UTP via suramin-sensitive $P2Y_2$ receptor on CF from adult rats is partially counteracted by synchronous activation of $P2Y_{11}$ receptors. UTP (100 µM) with or without suramin (30 µM, Ai), reactive blue-2 (RB-2, 30 µM, Aii), NF 340 (10 µM, Aiii) or Carbenoxolone (CBX 10 µM, B) were incorporated in culture media, and, therefore, contacted with the cells during the whole assay. In panel A and B, the ordinates represent changes in cell growth (MTT values) as compared to controls in the absence of test drugs at the same points (see Fig. 6). Zero represents similarity between the two values (drugs vs control); positive and negative values represent facilitation or inhibition of cell growth relative to control data obtained at the same time points. Each bar represents pooled data from an n number of individuals; four replicas were performed for each individual experiment. The vertical bars represent S.E.M.. *$p<0.05$ represent significant differences from control values obtained in the absence of test drugs. #$p<0.05$ represent significant differences from UTP (100 µM) alone. Panel C shows $P2Y_2$ and $P2Y_{11}$ receptors

immunoreactivity of adult rat CF cultured with UTP (100 μM) during 28 days. Confocal micrographs shown are representative of three independent experiments; scale bar is 100 μm.

After growing cells in culture during 28 days in the presence of UTP (100 μM), adult rat CF differentiate into stellate shaped myofibroblasts (see also Figure 4) which still express significant amounts of $P2Y_2$ and $P2Y_{11}$ receptors (Figure 12C). Cell processes of activated CF exhibit outstanding $P2Y_{11}$ receptor immunoreactivity, whereas the $P2Y_2$ receptor staining is sparser.

Cyclic AMP mediates the anti-proliferative effect of the $P2Y_{11}$ receptor in rat cardiac fibroblasts

Considering the involvement of the AC / cyclic AMP cascade on UTP-induced $[Ca^{2+}]_i$ responses operated by the $P2Y_{11}$ receptor in CF, we investigated the contribution of this pathway to the anti-proliferative effect of NF 546 using a similar experimental strategy. Figure 13 shows that the inhibitory effect of the selective $P2Y_{11}$ receptor agonist, NF 546 (10 μM), decreased consistently ($p<0.05$) upon blocking the activity of AC and EPAC respectively with SQ 22536 (30 μM, n=3) (Figure 13A) and ESI-09 (10 μM, n=3) (Figure 13B). Modification of the anti-proliferative action of NF 546 (10 μM) in the presence of the potent PKA inhibitor, H-89 (10 μM, n=3), was restricted to culture day 21 (Figure 13AC).

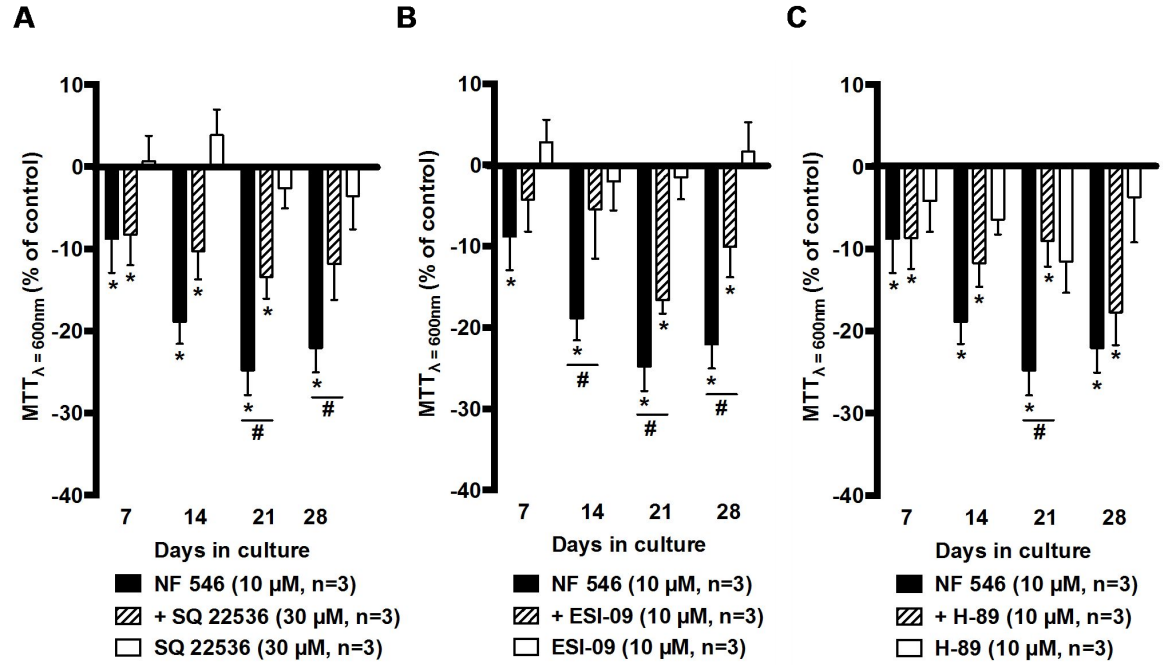

Figure 13 - Cyclic AMP mediates the anti-proliferative effect of the $P2Y_{11}$ receptor in rat cardiac fibroblasts. Shown is the variation of cell growth caused by continuous application of UTP (100 μM) to the culture medium in the absence and in the presence of inhibitors of AC, PKA and EPAC, respectively SQ 22536 (30 μM, A), H-89 (10 μM, B) and ESI-09 (10 μM, C). The ordinates

represent changes in MTT values compared to control cultures grown in the absence of test drugs at the same time points. Each column represents pooled data from n individuals; 4 replicates were performed for each individual experiment. The vertical bars represent S.E.M.. *p < 0.05 represent significant differences compared with the effect of UTP (100 μM) alone.

Discussion

Using selective agonists and antagonists of pyrimidine-sensitive P2Y purinoceptors, our results indicate that metabotropic $P2Y_2$ and $P2Y_4$ exert dominant roles on UTP-induced proliferation and $[Ca^{2+}]_i$ signaling, respectively, in cardiac myofibroblasts isolated from adult rat ventricles. While these findings agree with the previously described pro-fibrotic action of the nucleotide (Chen et al., 2012; Yitzhaki et al., 2006), we suggest here that this characteristic may be partially counteracted by synchronous activation of a previously unrecognized $P2Y_{11}$ receptor that is positively coupled to the AC / cyclic AMP / EPAC pathway in CF (see *e.g*. Kugelgen et al., 2006). The anti-proliferative action of the $P2Y_{11}$ receptor may contribute to the reported cardioprotective effect of this receptor against ischemia and reperfusion (Djerada et al., 2013), since in this condition high extracellular adenine and uracil nucleotide levels are detected (Wihlborg et al., 2006).

Notwithstanding the fact pyrimidine-sensitive $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors have been reported in cardiac myofibroblasts from neonatal rats (Talasila et al., 2009), there is a lack of information regarding the role of these receptors on cell proliferation and differentiation during a time (*e.g.* 28 days) required to operate remodeling changes in the heart *in vivo*. Moreover, data obtained in developmentally immature cells must be interpreted with caution when one wants to extrapolate the results to adulthood (Zhou and Dobrev, 2012). As a matter of fact, it has been shown that activation of $P2Y_2$ receptors up-regulates the proliferation of human CF (Chen et al., 2012) and that these receptors are involved in Ca^{2+} signaling in human valvular myofibroblasts (Liang et al., 2008). The pro-fibrotic effect of UTP, via the activation of $P2Y_2$ receptors, was also observed in adult rat CF and this effect was blunted in $P2Y_2^{-/-}$ mice (Braun et al., 2010). There are, however, reasons in the literature to believe that $P2Y_2$ receptor-mediated pro-fibrotic responses are transient (<24 h) and UTP effects may implicate ATP

release from stimulated cells and the interplay with other P2Y receptors, which pharmacological profile was explored in our study.

Intracellular calcium is essential for CF proliferation, migration and transdifferentiation into (activated) myofibroblasts. Moreover, abnormalities in Ca^{2+} handling by cardiac cells may determine arrhythmogenesis and heart fibrosis. We report here that adult rat CF respond to UTP by increasing $[Ca^{2+}]_i$ through the activation of fast desensitizing $P2Y_4$ receptors, which are co-expressed with $P2Y_2$ and $P2Y_{11}$ receptors that maintain their presence and activity in cells for weeks as demonstrated by immunofluorescence confocal microscopy, Western blot analysis and long-term (28 days) proliferation studies. The dominant role of the $P2Y_4$ receptor over $P2Y_2$ and $P2Y_{11}$ receptors on fast $[Ca^{2+}]_i$ signals operated by UTP was inferred by the rank order of potency of subtype-selective agonists: MRS 4062 > MRS 2768 ~ NF 546, respectively. Furthermore, attenuation of UTP-induced $[Ca^{2+}]_i$ transients by both suramin and reactive blue-2 also supports the involvement of the $P2Y_4$ receptor (Kugelgen et al., 2006). Participation of the $P2Y_6$ receptor, which could be activated by UDP resulting from the extracellular catabolism of UTP by ectonucleotidases, was discarded since UDP (100 μM) was the least potent agonist and no changes were verified on $[Ca^{2+}]_i$ transients produced by both UTP and UDP in the presence of MRS 2578 (Mamedova et al., 2004); it is worth to note that in this study MRS 2578 was applied in a higher concentration (300 nM) than that required to prevented activation of $P2Y_6$ receptors at the rat urinary bladder (Carneiro et al., 2014) and in human bone marrow stromal cells (Noronha-Matos et al., 2012).

Functional data shows that UTP and the $P2Y_4$ receptor agonist MRS 4062 trigger fast $[Ca^{2+}]_i$ responses, which desensitize very rapidly upon repeated agonist applications. Rapid mobilization of $[Ca^{2+}]_i$ seems to occur via the activation of a G_q protein-coupled receptor linked to the PLC/IP_3 pathway in CF. This conclusion is based on several lines of evidence, including the fact that (1) inhibition of PLC with U73122, (2) blockade of the IP_3 receptor with 2-APB, and (3) depletion of intracellular Ca^{2+}-stores with thapsigargin plus caffeine, all attenuated the fast $[Ca^{2+}]_i$ mobilization by UTP. Extracellular Ca^{2+} may also play some role to sustain the late component (phase II) of UTP-induced $[Ca^{2+}]_i$ rise, since its removal from the incubation medium significantly attenuated the nucleotide response.

Fibroblasts lack voltage-gated Ca^{2+} channels and Ca^{2+} influx in response to activation of G-protein-coupled receptors sensitive to UTP is more likely to occur predominantly via ligand-gated ion channels. Cooperation between metabotropic P2Y receptors and ionotropic P2X4 and P2X7 receptors could be one possibility to explain late $[Ca^{2+}]_i$ oscillations induced by UTP, since these receptor subtypes coexist in CF and possess unified pro-fibrotic actions (reviewed in Lu et al., 2014). The fact that ionotropic P2X receptors are almost exclusively activated by ATP and, thus, insensitive to uracil nucleotides, is apparently not a problem since we showed here that selective activation of the $P2Y_4$ receptor with MRS 4062 facilitates ATP translocation to the extracellular milieu in CF. Regrettably, we failed to block $[Ca^{2+}]_i$ responses produced by ATP (3-300 μM) in the presence of two highly selective and potent P2X4 and P2X7 receptor antagonists, respectively 5-BDBD (3-10 μM) and A 438079 (3-10 μM) (unpublished observations).

Crosstalk between UTP-sensitive P2Y receptors and other ligand-gated ion channels, such as TRPM7 and TRPC3, may have pathophysiological impact given that these receptors contribute to fibroblast-to-myofibroblast transition and cardiac arrhythmogenesis (reviewed in Zhou et al., 2012). Coincidently or not, the TRPM7 channel is activated by a cyclic AMP-dependent pathway and is blocked by 2-APB, when the latter compound is applied in the micromolar concentration range (reviewed in Chatelier et al., 2012). 2-APB also blocks all members of the TRPC family, which can be stimulated by diacylglycerol and act as store operated channels to compensate for depletion of intracellular Ca^{2+} stores (Parekh and Putney, 2005). These features fit in the results obtained in this study. However, most of the pharmacological tools available to study the activity of TRP channels lack specificity, thus preventing investigation of more definitive involvement of these channels on Ca^{2+} influx into CF besides our data showing that 2-APB (50 μM) depresses UTP-induced $[Ca^{2+}]_i$ plateau.

Another possibility to increase Ca^{2+} influx into cardiac myofibroblasts linked to activation of UTP-sensitive P2Y receptors is their interplay with the Na^+/Ca^{2+}-exchanger (NCX) operating in the reverse mode. Involvement of the NCX is possible because these cells express $Na_v1.5$ channels, which are responsible for larger and persistent Na^+ currents compared to those observed in cardiomyocytes (Chatelier et al., 2012). NCX has been shown to contribute to intracellular Ca^{2+} overload in cardiomyocytes during myocardial infarction (Chao and Xia, 2010), a

situation that favors adenine and uracil nucleotides overflow into the extracellular milieu (Wihlborg et al., 2006). Notwithstanding this possibility, selective inhibition of NCX with KB-R7943 (Lailov et al., 1999) or ORM-10103 (Jost et al., 2013) did not affect UTP-induced $[Ca^{2+}]_i$ signals in rat CF.

Involvement of an AC / cyclic AMP pathway on late $[Ca^{2+}]_i$ oscillations was hypothesized because stimulation of CF with UTP concentration-dependently increased intracellular cyclic AMP levels. Moreover, we showed that both reversible and irreversible inhibition of the AC activity respectively with SQ 22536 and MDL12,330a impacted on the magnitude of UTP-induced $[Ca^{2+}]_i$ plateau. Considering that the $P2Y_{11}$ receptor is the only P2 receptor subtype positively coupled to AC (Balogh et al., 2005; Amisten et al., 2007), accumulation of cyclic AMP points towards the concurrent activation of this receptor as a consequence of CF stimulation by UTP. In agreement with this hypothesis data demonstrated that the inhibitory effects of SQ 22536 and MDL12,330a were very similar to that obtained with the selective $P2Y_{11}$ receptor antagonist, NF340.

Cellular effects operated by cyclic AMP have been mostly attributed to the activation of PKA. However, novel cyclic AMP targets have emerged to explain cell responses that are insensitive to PKA inhibition (reviewed in Dekkers et al., 2013). The exchange protein directly activated by cAMP (EPAC) is a cyclic AMP-regulated guanine nucleotide exchange factor (GEF) that favors GDP/GTP exchange and, thereby, activation of small Ras-like GTPases, such as Rho, Rac and Ras. Interestingly, EPAC is known to control distinct cellular responses, including calcium handling, cell proliferation, differentiation, migration, fibrogenic and inflammatory responses. In the present study, we show that inhibition of EPAC with ESI-09, but not PKA with H-89, decreased UTP-induced $[Ca^{2+}]_i$ oscillations and attenuated the anti-proliferative effect of the $P2Y_{11}$ agonist, NF 546. This is not surprising since data in the literature indicate that EPAC and PKA differentially inhibit specific fibroblast functions (Dekkers et al., 2013). For instance, activation of PKA inhibits collagen synthesis by inhibiting the pro-fibrotic protein kinase C-δ, whereas activation of EPAC inhibits fibroblast growth by activating Rap1 and subsequent inhibition of extracellular signal-regulated kinases (ERK) in the airways. Notwithstanding, both PKA and EPAC contribute to inhibit fibroblast transdifferentiation to myofibroblasts induced by several G-protein-coupled

receptors. Investigation of all putative downstream effectors of the EPAC pathway in CF, which include phospholipase C-ε, phospholipase D, extracellular signal-regulated kinases (ERK1/2), phosphoinositide 3-kinase-dependent protein-kinase B (PKB)/Akt, and nuclear factor (NF)-κB (reviewed in Dekkers et al., 2013), is still lacking yet it is certainly beyond the scope of this study. Alternatively, the $P2Y_{11}$ receptor can contribute to sustain $[Ca^{2+}]_i$ by favoring cyclic adenosine diphosphate ribose (cADPR) production and subsequent ADP-ribosylcyclase (ADPRC) activation, which catalyzes the synthesis of various molecules endowed with Ca^{2+} mobilizing activity (Moreschi et al., 2008), but this also needs further investigations.

To our knowledge this is the first time that the UTP-sensitive $P2Y_{11}$ receptor has been involved in Ca^{2+} influx from the extracellular milieu required to sustain the plateau phase of $[Ca^{2+}]_i$ response in CF. Other authors reported that ATP and UTP equally increased $[Ca^{2+}]_i$ in 1321N1 neuroblastoma cells transfected with the human $P2Y_{11}$ receptor (Communi et al., 1999), but the two nucleotides diverge on the signaling pathways recruited to increase $[Ca^{2+}]_i$ even though they might be acting on the same receptor population (Balogh et al., 2005; Amisten et al., 2007). Synergism between $P2Y_4$ and $P2Y_{11}$ receptors to increase $[Ca^{2+}]_i$ in CF may be explained either by synchronous activation of both receptors coupled to distinct signaling pathways or by the involvement of UTP-induced ATP release (Kringelbach et al., 2014) leading to subsequent $P2Y_{11}$ receptor activation. Our findings suggest that AC and PLC act independently in a cooperative manner to sustain elevated $[Ca^{2+}]_i$ levels during the plateau (phase II) of the UTP response. Release of ATP and autocrine positive feedback through $P2Y_{11}$ receptors has been demonstrated in macrophages activation by lipopolysaccharide (Sakaki et al., 2013). CF can release ATP via connexin hemichannels) (Lu et al., 2012) and the release of the nucleotide has been involved in $[Ca^{2+}]_i$ signaling cooperation between P2Y and other metabotropic receptors (e.g. histamine H_1, bradykinin B_2) in human fibroblasts of the subcutaneous tissue (Pinheiro et al., 2013a; Pinheiro et al., 2013b). Data from this study show that activation of the $P2Y_4$ receptor with MRS 4062 facilitates the translocation of ATP to the extracellular milieu. Involvement of hemichannels containing Cx43 in ATP release was inferred (1) in confocal microscopy and Western blot studies, demonstrating that CF exhibit Cx43 immunoreactivity, and (2) in functional studies, showing that blockage of Cx43-containing hemichannels with CBX and H-1152 decreased both the release of

ATP and the magnitude of late $[Ca^{2+}]_i$ signals produced by UTP. Interestingly, the use of connexin-blocking antibodies can disrupt both myocyte-fibroblast and fibroblast-fibroblast communication aside from electrical regulation (Baudino et al., 2008). A better understanding of these interactions may elucidate if ATP release via Cx43-containing hemichannels also play a role in cell-to-cell signaling and cardiac remodeling.

Proliferation and migration of CF are key events in the fibrotic response of cardiac remodeling, and extracellular nucleotides are known to have mitogenic properties in several types of cells. Our data also demonstrate that adult rat CF respond to UTP, but no to UDP, by increasing cell growth through the activation of $P2Y_2$ receptors. This was suggested because the selective $P2Y_2$ receptor agonist, MRS 2768 (Ko et al., 2008), mimicked the proliferative effect of UTP, whereas activation of the $P2Y_4$ receptor with MRS 4062 (Meis et al., 2010) was without effect on long-term CF growth evaluation. Differences between the blocking potency of suramin and reactive blue-2, with the latter being a weaker $P2Y_2$ receptor antagonist than suramin (Kugelgen et al., 2006), strengthen our assumption that UTP favors proliferation of rat CF preferentially via $P2Y_2$ receptor activation. The lack of a $P2Y_4$ receptor-mediated effect on CF growth is not surprising given that we show here that this receptor desensitizes rapidly upon repeated applications or prolonged contact with its agonists, either UTP or MRS 4062. Normalization of type I collagen production by the number of viable cells (MTT assay) indicates that UTP exerts a preferential effect on cell growth rather than in extracellular matrix deposition. Interestingly, the overall effect of UTP on CF growth was balanced by synchronous activation of $P2Y_{11}$ receptors and this was concluded because selective blockade of this receptor with NF 340 (Meis et al., 2010) potentiated growth of CF caused by long-term exposure to the nucleotide. The anti-proliferative effect of the $P2Y_{11}$ receptor was confirmed using the selective agonist of this receptor, NF 546, which significantly decreased CF growth under similar experimental conditions.

Conclusion

In conclusion, our findings show that UTP-sensitive $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors differentially affect $[Ca^{2+}]_i$ signals and proliferation of rat CF. Activation of

the $P2Y_4$ receptor is the main responsible for UTP-induced $[Ca^{2+}]_i$ accumulation, an effect that was prolonged by synchronous activation of the $P2Y_{11}$ receptor by ATP released through Cx43-containing hemichannels leading to Ca^{2+} influx from the extracellular milieu. More importantly, data from this study show that long-term activation of the $P2Y_{11}$ receptor counteracts the proliferating effect of UTP operated by the $P2Y_2$ receptor on cultured CF. The anti-proliferative property of the novel $P2Y_{11}$ receptor seems to be mediated by downstream activation of the AC / cyclic AMP / EPAC pathway. This property, together with the prominent action of the $P2Y_{11}$ receptor in controlling cardiomyocyte contractility may point towards this receptor being an important target for therapeutic intervention to prevent deleterious cardiac remodeling under various pathological conditions.

PAPER 2

(submitted for publication)

ADP-induced Ca^{2+} signaling and proliferation of rat ventricular myofibroblasts depend on phospholipase C-linked TRP channels activation within lipid rafts

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Abstract

Nucleotides released during heart injury affect myocardium electrophysiology and remodelling through P2 purinoceptors activation in cardiac fibroblasts (CF). ATP and UTP endorse $[Ca^{2+}]_i$ accumulation and growth of DDR-2/(-SMA-expressing myofibroblasts from adult rat ventricles via $P2Y_4$ and $P2Y_2$ receptors activation, respectively. Ventricular myofibroblasts also express ADP-sensitive $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors as demonstrated by immunocytochemistry and western blot analysis, but little information exists on ADP effects in these cells. ADP (0.003-3mM) caused fast $[Ca^{2+}]_i$ transients originated from thapsigargin-sensitive internal stores, which partially declined to a plateau sustained by capacitative Ca^{2+} entry through transient receptor potential (TRP) channels that can be inhibited by 2-APB (50μM) and flufenamic acid (100μM). Hydrophobic interactions between $G_{q/11}$-coupled P2Y purinoceptors and TRP channels were suggested by prevention of the ADP-induced $[Ca^{2+}]_i$ plateau after PIP_2 depletion with LiCl (10mM) and removal of cholesterol from lipid rafts with methyl-ß-cyclodextrin (2mM). ADP $[Ca^{2+}]_i$ transients were insensitive to $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor antagonists, MRS2179 (10μM), AR-C66096 (0.1μM) and MRS2211 (10μM), respectively, but were attenuated by suramin and reactive blue-2 (100 μM) which also blocked $P2Y_4$ receptors activation by UTP. CF growth and type I collagen production were favoured upon activation of MRS2179-sensitive $P2Y_1$ receptors with ADP or ADPßS (30 μM). In conclusion, ADP exerts a dual role on ventricular myofibroblasts: $[Ca^{2+}]_i$ transients are mediated by fast-

desensitizing $P2Y_4$ receptors, whereas the pro-fibrotic effect of ADP involves the $P2Y_1$ receptor. Data also show that ADP-induced capacitative Ca^{2+} influx depends on phospholipase C-linked TRP channels opening in lipid raft microdomains.

Introduction

Cardiac fibroblasts (CF) outnumber cardiomyocytes in adult hearts (Porter and Turner, 2009). Due to their abundance, strategic localization and remarkable potential for activation, CF may serve as sentinel cells that sense myocardial injury and trigger reparative responses (Shinde and Frangogiannis, 2014). These cells contribute to maintain extracellular matrix (ECM) homeostasis and to distribute mechanical forces throughout the myocardium; CF also serve as an electrical barrier between atria and ventricles in order to facilitate proper cardiac contraction (Camelliti et al., 2005; Zeisberg and Kalluri, 2010). Mounting evidences demonstrate that upon activation by mechanic, noxious and inflammatory stimuli resident quiescent CF transdifferentiate into myofibroblasts, a fibroblast-smooth cell hybrid that more effectively secretes growth factors, cytokines and other signalling molecules through mechanisms mostly driven by intracellular Ca^{2+} transients. Besides the paracrine effects of cardiac myofibroblasts, disruption of direct cell-cell communication can lead to dysregulation of biochemical, mechanical and electrical signals to neighbouring cardiomyocytes (Baudino et al., 2008). Thus, involvement of CF in myocardial dysfunction and arrhythmogenesis makes these cells natural targets in cardiac remodelling, yet the molecular signals and intracellular pathways underlying their activation, proliferation and differentiation remain incompletely understood.

During stressful conditions, such as cardiac ischemia/reperfusion and myocardium overload, ATP and UTP are released in huge amounts into the extracellular milieu (Wihlborg et al., 2006) favouring the activation of multiple P2 purinoceptor subtypes (Burnstock, 2006). The extent of P2 purinoceptors-mediated actions by adenine and uracil nucleotides may be limited by the presence of membrane-bound ecto-nucleotidases, which sequentially degrade nucleoside 5'-triphosphates to their respective nucleoside 5'-di- and monophosphates (Yegutkin, 2008; Cardoso et al., 2015). Besides the well-recognized roles of ATP and its end-product, adenosine, few reports have questioned the action of biologically-active intermediate metabolites, such as ADP, in cardiac function and remodelling. ADP is a preferential activator of G-protein-coupled $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors (Burnstock, 2006), but the nucleotide may also activate the UTP-preferring $P2Y_4$ receptor (Bogdanov et al., 1998)

implicated on fast $[Ca^{2+}]_i$ transients in cardiac myofibroblasts (Certal et al., 2015). While $P2Y_1$ and $P2Y_4$ receptors couple to the phospholipase C / IP_3 / diacylglycerol (DAG) pathway via a $G_{q/11}$ protein anchor, the $P2Y_{12}$ and $P2Y_{13}$ receptors most frequently inhibit adenylate cyclise activity through $G_{i/o}$ protein coupling (Alexander et al., 2015).

The presence of $P2Y_1$, $P2Y_4$ and $P2Y_{13}$ receptor transcripts has been demonstrated in neonatal rat cardiac fibroblasts by reverse transcription-polymerase chain reaction (RT-PCR) and/or Northern blot analysis (Zheng et al., 1998; Talasila et al., 2009). In a recent study, we demonstrated by immunofluorescence confocal microscopy and Western blot analysis that CF from adult rat ventricles express UTP-sensitive $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors, which activation differently regulates $[Ca^{2+}]_i$ signalling and cells growth (Certal et al., 2015). However, little information exists on the role of ADP-preferring P2Y receptors in CF physiology.

Assembly of metabotropic purinergic receptors and signalling molecules is highly dependent on specialized membrane microdomains (lipid rafts, raft-like structures, caveolae) (D'Ambrosi and Volonté, 2013). These specialized membrane structures enclose complex control systems involving molecular association, cooperation, conformational or electronic state changes of enzymes, receptors and channels. Caveolae are 50- to 100-nm omega-shaped invaginations at the plasma membrane formed by polymerization of caveolins, hairpin-like palmitoylated integral membrane proteins from lipid rafts that tightly bind cholesterol (Williams and Lisanti, 2004). Caveolin-1 is the principal structural component of caveolae in CF and can be used as a marker of these structures (Gratton et al., 2004). Caveolin-binding motifs are present in many caveolae-associated proteins, like G proteins, protein kinase C (PKC), ion channels and transporters. Binding to caveolin is able to segregate cellular processes during signal transduction by promoting isolated assembly of membrane protein superstructures and to focus signalling input by accumulation of specific receptors within lipid raft/caveolae microdomains. It is well known that P2 purinoceptors (in particular metabotropic $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$, but also $P2Y_{13}$), as well as ectonucleotidases and nucleotide transporters, localize in lipid rafts/caveolae in many cell types (Garcia-Marcos et al., 2009; reviewed in D'Ambrosi and Volonté, 2013), but no data is available concerning CF.

This study was designed to investigate the role of ADP on $[Ca^{2+}]_i$ signalling and long-term growth of cultured CF from adult rat ventricles using subtype-selective agonists and antagonists of $P2Y_1$, $P2Y_4$, $P2Y_{12}$ and $P2Y_{13}$ receptors. We also attempted to investigate the signalling pathways implicated in ADP-induced $[Ca^{2+}]_i$ oscillations, cell proliferation and type I collagen production by CF using enzymatic inhibitors and subtype-specific ion channel blockers. The impact of lipid raft/caveolae organization of the plasma membrane in the lateral assembly between ADP-sensitive P2Y receptors and corresponding signalling pathways was evaluated using methyl-ß-cyclodextrin, which depletes cholesterol from plasma membrane lipid rafts.

Methods

Drugs and solutions

Adenosine 5′-diphosphate sodium salt (ADP), adenosine 5'-(α,β-methylene) diphosphate (ADPßS), 2-aminoethoxydiphenylborane (2-APB), bovine serum albumin (BSA), bromophenol blue, caffeine, 2-mercaptoethanol, methyl-β-cyclodextrin, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (thiazolyl blue formazan – MTT), direct red 80, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), fetal bovine serum (FBS), flufenamic acid, glycerol, phosphate buffered saline system (PBS), picric acid, protease inhibitor cocktail, reactive blue-2 (RB-2), suramin sodium salt, sodium deoxycholate, sodium dodecyl sulphate (SDS), trypsin, Tween 20, type I collagenase, uridine 5′-triphosphate (UTP), and cell culture reagents were from Sigma-Aldrich (St. Louis, USA). 2-(propylthio)adenosine-5'-O-(ß,γ-difluoro methylene) triphosphate tetrasodium salt (AR-C66096), 1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride (Chelerytrine), 2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea mesylate (KB-R7943), cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL 12,330a), 2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphornooxy)methyl]-4-pyridine carboxaldehyde disodium salt (MRS2211), 2'-deoxyN^6-methyladenosine 3',5'-biphosphate tetrasodium salt (MRS2179), [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo [3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365), thapsigargin, 1-[6-[[(17β)-

3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536) were obtained from Tocris Cookson Inc. (Bristol, UK). LiCl was obtained from Merck (Darmstadt, Germany). Fluo-4 NW dye was supplied by Molecular Probes (Eugene, OR, USA). U73122, thapsigargin and SQ 22536 were dissolved in dimethylsulphoxide (DMSO). The other drugs were dissolved in distilled water. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate controls were done. DMSO (<0.05% v/v) did not affect the parameters under study.

Animals

Wistar rats (150-200 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21 ºC) and a regular light (06.30 –19.30 h) - dark (19.30–06.30 h) cycle, with food and water ad libitum. Animal handling and experiments were in accordance with the guidelines prepared by Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010).

Cell cultures

Rat cardiac fibroblasts were isolated from the ventricles of adult Wistar rats by the explant technique in which fibroblasts migrate from minced tissue and grow in fibroblast growth medium (Certal et al., 2015). Cells were cultured in DMEM medium supplemented with 15% fetal bovine serum (FBS), 1% of amphotericin B and 1% of penicillin/streptomycin, at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. Medium was replaced twice a week. Primary cultures were maintained until near confluence (~3-4 weeks), then adherent cells were enzymatically released with 0.04% trypsin-EDTA solution plus 0.025% type I collagenase in phosphate-buffered saline (PBS). The resultant cell suspension was cultured and maintained in the same conditions mentioned above.

Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were measured with the calcium sensitive dye Fluo-4 NW (Molecular Probes, Eugene, OR, USA) in a multi detection microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA) (Certal et al., 2015). Briefly, rat cardiac fibroblasts were seeded in flat bottom 96 well plates (Costar, Corning Inc., NY, USA) at a density of $3x10^4$ cells/mL. Cells were cultured for 7 days in supplemented DMEM as described before. On the day of the experiment, cells were washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 11.2 mM glucose, pH 7.4) and incubated at 37ºC for 45 minutes with the cell-permeant fluorescent Ca^{2+} indicator, Fluo-4 NW (2.5 µM), in 1x HBSS, 20 mM HEPES and 2.5 mM probenecid. After removal of the fluorophore loading solution, cells were washed again twice and 150 µL of Tyrode's solution was added per culture well. For the recordings, temperature was maintained at 32 ºC and readings were made with 5 s of interval, during approximately 30 min, using a tungsten halogen lamp. Fluorescence was excited at 485/20 nm and emission was measured at 528/20 nm. Calcium measurements were calibrated to the maximal calcium load produced by the calcium ionophore, ionomycin (5 µM, 100% response) (Certal et al., 2015; Pinheiro et al., 2013a; Pinheiro et al., 2013b).

Cell viability/proliferation

Viability/proliferation studies included the MTT assay as previously described (Certal et al., 2015; Pinheiro et al., 2013a; 2013b). Rat cardiac fibroblasts were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL and cultured in supplemented DMEM as described before. Cell cultures were routinely monitored by phase contrast microscopy and characterized at days 1, 7, 14, 21 and 28. The MTT assay consists on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan reaction product by viable cells. In the last 4h of each test period, cells were incubated with 0.5 mg/mL of MTT in the conditions referred above. The medium was carefully removed, decanted and the stained product dissolved with DMSO before absorbance (A) determination at 600 nm using a microplate reader spectrometer

(Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). Results were expressed as A/well.

Type I collagen determination

Type I collagen determination was performed using the Sirius Red staining assay (see Certal et al., 2015). Rat cardiac fibroblasts were cultured as described for the viability/proliferation studies. The staining protocol was adapted from Tullberg-Reinert and Jundt (2009). Cell layers were washed twice in PBS before fixation with Bouin's fluid for 1 h. The fixation fluid was removed by suction and the culture plates were washed by immersion in running tap water for 15 min. Culture dishes were allowed to air dry before adding the Sirius Red dye (Direct Red 80). Cells were stained for 1 h under mild shaking on a microplate shaker. To remove non-bound dye, stained cells were washed with 0.01 N hydrochloric acid and then dissolved in 0.1 N sodium hydroxide for 30 minutes at room temperature using a microplate shaker. Optical density was measured at 550 nm using 0.1 N sodium hydroxide as blank (Tullberg-Reinert and Jundt, 1999; Certal et al., 2015). Results were expressed as A/well.

Immunocytochemistry

Rat cardiac fibroblasts were seeded in chamber slides at a density of $2.5x10^4$ cells/mL and allowed to grow for 7-28 days. Cultured cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes, washed 3 times in PBS (10 minutes each) and, subsequently, incubated with blocking buffer I (10% FBS, 1% bovine serum albumin (BSA), 0.1% Triton X, 0.05% NaN_3) for 1 h. Primary antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN_3 in PBS), were applied [mouse anti-porcine vimentin 1:250 (DAKO); goat anti-human DDR2 1:25 (Santa Cruz); mouse anti human α-smooth muscle actin-FITC 1:250 (Sigma); rabbit anti-mouse $P2Y_1$ 1:50 (Alomone), rabbit anti-mouse $P2Y_{12}$ 1:100 (Alomone), rabbit anti-human $P2Y_{13}$ 1:25 (Alomone); mouse anti-human caveolin-1 1:400 (Abcam)] and the slides incubated overnight at 4ºC. After incubation, cells were washed 3 times in PBS 1X (10 minutes each). The donkey anti-rabbit IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 488, donkey anti-goat IgG Alexa Fluor 633 and anti-guinea pig IgG Alexa Fluor 568 secondary antibodies (Invitrogen) were diluted in blocking buffer II and applied for 1h at dark.

Cross-reactivity of the secondary antibodies was tested in control experiments in which primary antibodies were omitted. Finally, a last wash was performed with PBS 1X and glass slides were mounted with VectaShield medium and stored at 4ºC. Observations were performed and analysed with a laser scanning confocal microscope (Olympus FV1000, Olympus, Tokyo, Japan) (Noronha-Matos et al., 2012; Pinheiro et al., 2013a; Pinheiro et al., 2013b; Certal et al., 2015).

SDS-PAGE and Western blotting

Cardiac fibroblasts were homogenized in a lysis buffer with the following composition: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton-X-100m 0.1% SDS and protease inhibitor cocktail. Protein content of the samples was evaluated using the BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Samples were solubilized in SDS reducing buffer (0.125 mM Tris-HCl, 4% SDS, 0.004% bromophenol blue, 20% glycerol, and 10% 2-mercaptoethanol, pH 6.8 at 70 ºC for 10 min), subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (Millipore, MA, USA). Protein loads were 100 µg for $P2Y_1$, $P2Y_{12}$ e $P2Y_{13}$ receptors. The membranes were blocked for 1 h in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 + 5% BSA. Membranes were subsequently incubated with rabbit anti-mouse $P2Y_1$ 1:200 (Alomone), rabbit anti-mouse $P2Y_{12}$ 1:200 (Alomone) and rabbit anti-human $P2Y_{13}$ 1:200 (Alomone) in the above blocking buffer overnight at 4 ºC. Membranes were washed three times for 10 min in 0.1% Tween 20 in TBS and then incubated with donkey anti-rabbit IgG (HRP) (1:30000) (Abcam) and donkey anti-goat IgG (HRP) (1:25000) (Abcam) secondary antibody, for 60 min at room temperature. For comparison purpose, the membranes were also incubated with rabbit anti-human ß-actin 1:2500 (Abcam) and rabbit anti-human ß-tubulin 1:2500 (Abcam) antibodies following the procedures described above. Membranes were washed three times for 10 min and antigen-antibody complexes were visualized with the Immun-Star WesternC Chemiluminescent Kit (Bio-Rad Laboratories) using the ChemiDoc MP imaging system (Bio-Rad Laboratories).

Presentation of data and statistical analysis

Concentration-response curves were fitted by non-linear regression using GraphPad Prism 5.04 software (La Jolla, CA, USA) function: log(drug) vs. response. We assumed that data share best-fit values from top and bottom and a hill slope equal to 1. Fitting used the least squares method. Data are expressed as mean ± S.E.M. from an n number of individuals. Data from different individuals performed in triplicate were evaluated by one-way analysis of variance (ANOVA). Statistical differences found between control and drug-treated cultures were determined by the Bonferroni's method. P values < 0.05 (two-tailed) were considered to represent significant differences.

Results

ADP causes a biphasic effect on intracellular Ca^{2+} ($[Ca^{2+}]_i$) in myofibroblasts from adult rat ventricles

Figure 14A shows that cells cultured from adult rat ventricle explants exhibit positive immunoreactivity against both vimentin (Figure 14Ai; Agocha et al., 1997) and discoidin domain receptor 2 (DDR2) (Figure 14Ai and 14Aii; Morales et al., 2005; Goldsmith et al., 2010), which are reliable cardiac fibroblast-cell markers. These cells also stained positively for α-smooth muscle actin (α-SMA), a myofilament protein that is typically expressed by activated cardiac fibroblasts (myofibroblasts) (Figure 14Aii; Brown et al., 2005; Davis and Molkentin, 2014).

Incubation with ADP (100 μM) caused a fast (within seconds) $[Ca^{2+}]_i$ rise (phase I) in cardiac myofibroblasts, which attained 32±1% of the maximal calcium load produced by ionomycin (5 μM, 100% response); beyond this point, $[Ca^{2+}]_i$ levels declined to a plateau of elevated $[Ca^{2+}]_i$ (phase II) that remained fairly constant (Figure 14B and 14C). The initial $[Ca^{2+}]_i$ rise (phase I) produced by ADP decreased ($p<0.05$) in the presence of the selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (5 μM, n=4) (Thastrup et al., 1990), which is known to accelerate the depletion of intracellular Ca^{2+} stores when applied together with internal Ca^{2+} mobilizing agents, such as caffeine (10 mM) (Figure 14B). Removal of external Ca^{2+} (plus EGTA, 100 μM) significantly ($p<0.05$) depressed the late component of ADP (100 μM, n=4) response (phase II), keeping unaltered the initial fast rise (Figure 14C).

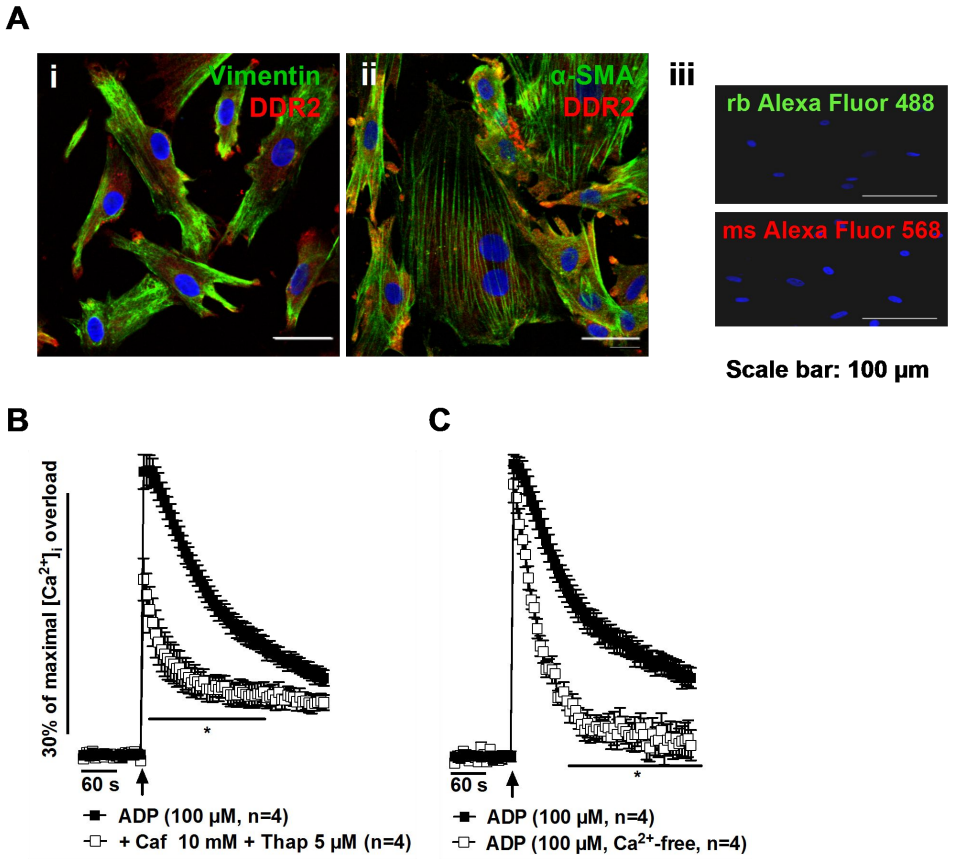

Figure 14 - ADP causes a biphasic effect on intracellular Ca^{2+} ($[Ca^{2+}]_i$) in myofibroblasts from adult rat ventricles Panel A shows that cells exhibited positive immunoreactivity against vimentin, which has been described as a reliable fibroblast marker (green, Ai), α-smooth muscle actin (α-SMA), a myofilament protein that typically is expressed by activated fibroblasts (myofibroblasts) (green, Aii) and discoidin domain receptor 2 (DDR2), a collagen specific receptor tyrosine kinase that is the most specific marker for cardiac fibroblasts and myofibroblasts (red, Ai and Aii). Negative controls, in which the cells were incubated only with the secondary antibodies labelled with Alexa Fluor 488 (green) and Alexa Fluor 568 (red) dyes, are shown in Aiii. Nuclei are stained with DAPI, which was included in the mounting medium. Scale bar 100 µm. Panels B and C represent $[Ca^{2+}]_i$ oscillations produced by ADP (100 µM) in cultured myofibroblasts from adult rat ventricles loaded with the fluorescent calcium indicator, Fluo-4 NW (2.5 µM, see Methods) in control conditions and after depletion of intracellular Ca^{2+} stores with thapsigargin (5 µM) plus caffeine (10 mM) (B) or after removal of extracellular Ca^{2+} (Ca^{2+}-free medium plus EGTA, 100 µM, C). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Black arrows indicate the time of ADP (100 µM) application. Each point represents a pooled data from an *n* number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *$p < 0.05$ represent significant differences from ADP (100 µM) alone.

Characterization of the P2Y receptor mediating ADP-induced $[Ca^{2+}]_i$ transients in ventricular myofibroblasts

ADP is a preferential activator of G-protein-coupled $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors (Burnstock, 2006). The presence of all these receptors was confirmed in 7-day cultures of adult rat ventricular myofibroblasts by immunofluorescence confocal microscopy (Figure 15A). Figure 15B shows that ADP (0.001-3000 μM, EC_{50} = 121 μM) concentration-dependently increased $[Ca^{2+}]_i$ accumulation in these cells, but its effect was 1.5 log concentration units less potent than that observed with UTP (0.001-3000 μM, EC_{50} = 3 μM) in the same experimental conditions (Figure 15B; see Certal et al., 2015).

The highly potent $P2Y_1$ receptor agonist, MRS2365 (N-methanocarba-2MeSADP), which has the ability to discriminate $G_{q/11}$-coupled from G_i/o-linked ADP-sensitive $P2Y_{12}$ and $P2Y_{13}$ receptors (Chhatriwala et al., 2004), had a limited effect on $[Ca^{2+}]_i$ accumulation in ventricular myofibroblasts; MRS2365 caused an effect of less than 10% of the maximal Ca^{2+} load produced by ionomycin (5 μM) when it was applied at a concentration (10 μM) high above its EC_{50} value of 0.4 nM for $P2Y_1$ receptor-mediated effects (Figure 15C).

Pre-treatment of ventricular myofibroblasts with selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor antagonists, respectively MRS2179 (10 μM, n=6), AR-C66096 (0.1 μM, n=5) and MRS2211 (10 μM, n=4), did not modify the magnitude and profile of ADP-induced $[Ca^{2+}]_i$ transients (Figure 15D). These results suggest that ADP-preferring receptors, such as $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$, are not involved in $[Ca^{2+}]_i$ signals produced by ADP (100 μM) in cultured ventricular myofibroblasts from adult rats. Taking into consideration that the UTP-sensitive $P2Y_4$ receptor dominates purinergic $[Ca^{2+}]_i$ transients in cardiac myofibroblasts (Certal et al., 2015) and that ADP is able to stimulate this receptor (Bogdanov et al., 1998; Burnstock, 2012), we tested whether the $P2Y_4$ receptor was mediating ADP $[Ca^{2+}]_i$ responses. As a matter of fact, $[Ca^{2+}]_i$ accumulation caused by ADP (100 μM) was attenuated ($p<0.05$) by suramin (100 μM, n=5) and reactive blue-2 (100 μM, n=5), with the latter being more potent than the former (Figure 15E-G). A similar antagonist profile was observed when UTP (3 μM) was used instead of ADP (100 μM) (Certal et al., 2015), thus strengthening our assumption that ADP-induced $[Ca^{2+}]_i$ transients were indeed operated by the $P2Y_4$ receptor.

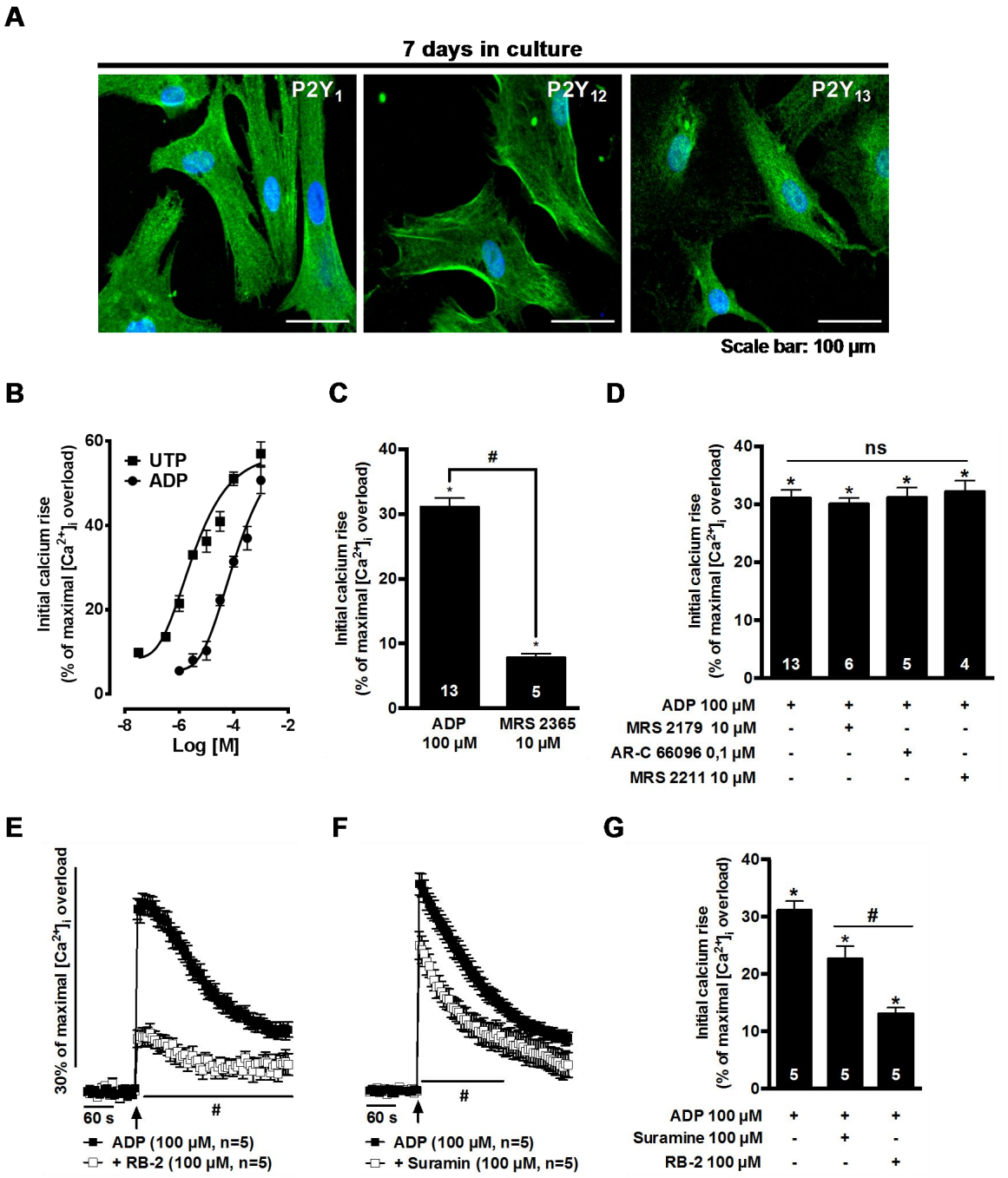

Figure 15 - Despite the presence of ADP-preferring $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors in ventricular myofibroblasts, $[Ca^{2+}]_i$ transients induced by ADP (100 µM) were predominantly mediated by $P2Y_4$ receptors activation. Panel A shows immunofluorescence confocal micrographs of fibroblasts from adult rat ventricles grown in culture for 7 days stained against $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors. Images are representative of three independent experiments; scale bar is 100 µm.. Panel B shows concentration-response curves of $[Ca^{2+}]_i$ oscillations produced by UTP (0.03-1000 µM) and ADP (1-1000 µM). Panel C compares the magnitude of initial $[Ca^{2+}]_i$ rises produced by ADP (100 µM) and the selective $P2Y_1$ receptor agonist, MRS 2365 (10 µM). Panel D compares the magnitude of $[Ca^{2+}]_i$ peaks produced by ADP (100 µM) applied either in the absence or in the presence of selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors antagonists, respectively MRS 2179 (300 nM), AR-C66096 (0.1 µM), and MRS 2211 (10 µM). Panels E-G show the blocking effects of two non-selective P2 receptor antagonists, reactive blue-2 (RB-2, 100 µM) and suramin (100 µM), on ADP (100 µM)-induced $[Ca^{2+}]_i$ oscillations. In all the experiments $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Each point/bar represents pooled data from an *n* number of individuals; experiments were performed in triplicate. The vertical

bars represent S.E.M.. *p < 0.05 represent significant differences from control values obtained in the absence of test drugs, #p<0.05 represent significant differences from ADP (100 μM) alone.

ADP-induced fast $[Ca^{2+}]_i$ rise (phase I) depends on activation of phospholipase C, but it is protein kinase C independent

The $P2Y_4$ receptor couples via $G_{q/11}$-protein to phospholipase C releasing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from the plasma membrane, which favour Ca^{2+} recruitment from internal stores and protein kinase C (PKC) activation, respectively. Among the ADP-preferring P2Y receptors, only the $P2Y_1$ subtype shares a common transduction pathway with the $P2Y_4$ receptor, whereas both $P2Y_{12}$ and $P2Y_{13}$ receptor subtypes are $G_{i/o}$-linked leading to the inhibition of adenylate cyclase activity (Alexander et al., 2015). Pharmacological inhibition of these transduction pathways may offer additional inputs to discriminate the receptors involved in ADP-induced $[Ca^{2+}]_i$ response in these cells. Selective inhibition of phospholipase C (PLC) activity with U73122 (3 μM, n=5) decreased ($p<0.05$) both the initial rise (phase I) and the plateau (phase II) of ADP (100μM)-induced $[Ca^{2+}]_i$ response in ventricular myofibroblasts (Figure 16A), but the same was not observed after pre-treatment of these cells with selective adenylate cyclase inhibitors, such as SQ22536 (30 μM, n=6, Figure 16B) and MDL12,330a (10 μM, n=2, data not shown). Inhibition of PKC activity with chelerythrine (3 μM) also failed to change the magnitude and shape of $[Ca^{2+}]_i$ transients produced by ADP (100 μM) (Figure 16C).

Capacitative Ca^{2+} entry through transient receptor potential (TRP) channels is responsible for the $[Ca^{2+}]_i$ plateau (phase II) produced by ADP

Phospholipase C activated downstream $G_{q/11}$-coupled receptors stimulation is the main modulator of Ca^{2+}-permeable transient receptor potential (TRP) channels. TRP channels are a family of six-transmembrane domain proteins that are regulated by phosphoinositides, such as PtIns(4,5)P2 (PIP_2) and IP_3 (reviewed in Ramsey et al., 2006), with certain subtypes (TRPC2, TRPC3, TRPC6 and TRPC7) being also activated by DAG independently of PKC stimulation (Harteneck and Gollasch, 2011). Our results show that the plateau (phase II) of ADP-induced $[Ca^{2+}]_i$ accumulation requires Ca^{2+} influx from the extracellular milieu and is dependent on PLC, but not PKC, activation, which led us to hypothesize the

participation of TRP channels as mediators of ADP-induced capacitative Ca^{2+} entry.

Although the pharmacology of most TRP channels is poorly developed and the effects reported are often complex, broad spectrum agents, such as 2-aminoethoxydiphenylborane (2-APB), have been instrumental to explore the participation of these channels in diverse experimental conditions (Ramsey et al., 2006; Harteneck and Gollasch, 2011). The late component of ADP (100 μM)-induced $[Ca^{2+}]_i$ response (phase II) was significantly ($p<0.05$) depressed in the presence of 2-APB (50 μM, n=5), which did not affect the initial fast $[Ca^{2+}]_i$ rise (Figure 16D). Likewise, flufenamic acid (100 μM), a non-selective blocker of TRP channels (Guinamard et al., 2013), decreased ($p<0.05$) ADP-induced $[Ca^{2+}]_i$ plateau in ventricular myofibroblasts (Figure 16E).

Inhibition of inositol monophosphatase with LiCl (10 mM), thus preventing the synthesis of phosphoinositides (Hanson, 1991) and the regeneration of adequate PIP_2 levels to be hydrolyzed to IP_3 and DAG (Berridge and Irvine, 1989), also significantly ($p<0.05$) reduced the second (plateau) phase of $[Ca^{2+}]_i$ response produced by ADP (100 μM) in ventricular myofibroblasts (Figure 16F).

Ca^{2+} influx through Na^+/Ca^{2+} exchanger (NCX) operating in the reverse mode does not account for ADP-induced $[Ca^{2+}]_i$ accumulation

Another possibility to increase Ca^{2+} influx linked to activation of metabotropic P2Y receptors is the putative interplay of these receptors with the Na^+/Ca^{2+} exchanger (NCX) operating in the reverse mode, which can be energized by Na^+ influx through $Na_v1.5$ channels presented in cardiac fibroblasts (Chatelier et al., 2012). Notwithstanding this possibility, preferential inhibition of NCX with KB-R7943 (50 μM) (Ladilov et al., 1999), did not significantly ($p>0.05$) affect ADP (100 μM)-induced $[Ca^{2+}]_i$ transients in ventricular myofibroblasts (Figure 16E) and the same occurred when UTP was used instead of ADP (Certal et al., 2015).

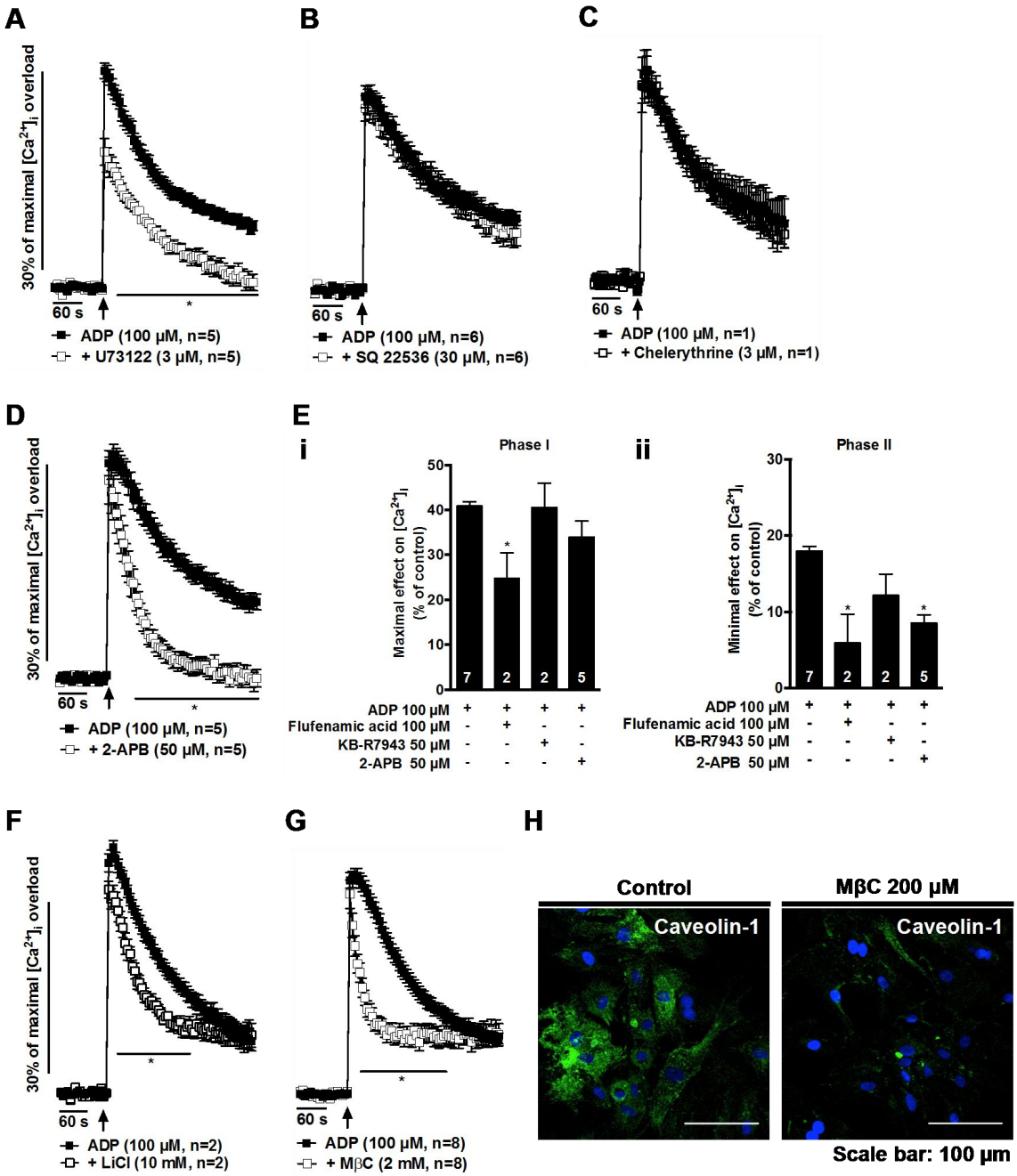

Figure 16 - Mechanisms underlying ADP-induced biphasic $[Ca^{2+}]_i$ response by cultured ventricular fibroblasts. Panels A-C show that ADP-induced fast $[Ca^{2+}]_i$ rises (phase I) depend on phospholipase C (PLC) activity, but are independent on adenylate cyclase (AC) and protein kinase C (PKC) activation. Shown are the effects of ADP (100 µM) obtained in the absence and in the presence of U73122 (3 µM, A, a PLC inhibitor), SQ 22536 (30 µM, B, an AC inhibitor) and chelerythrine (3 µM, C, a PKC inhibitor). Panels D and E show that the capacitative Ca^{2+} entry through transient receptor potential (TRP) channels is responsible for the late $[Ca^{2+}]_i$ plateau (phase II) of the ADP (100 µM) response. Shown are the effects of ADP (100 µM) obtained in the absence and in the presence of two TRP channel inhibitors, 2-APB (50 µM), and flufenamic acid (100 µM), and KB-R7943 (50 µM) that inhibits the Na^+/Ca^{2+} exchanger working in the reverse mode. Phase I was considered during the first 150 seconds after ADP application, while phase II was calculated thereafter. Panels F and G represent $[Ca^{2+}]_i$ oscillations produced by ADP (100 µM) in the absence and in the presence of signalling disruptors by acting on membrane lipids, namely the inositol monophosphatase inhibitor, LiCl (10 mM, F), and methyl-β-cyclodextrin (MβC, 2 mM, G), which extracts cholesterol from the plasma membrane leading to decreases in caveolin-1 content (see Panel H; immunofluorescence confocal micrographs shown are representative of two

independent experiments; scale bar is 100 μm). Black arrows indicate the time of ADP (100 μM) application. Each point/bar represents pooled data from an n number of individuals; experiments were performed in triplicate. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from ADP (100 μM) alone.

ADP-induced $[Ca^{2+}]_i$ plateau (phase II) requires hydrophobic interactions between P2Y receptors and TRP channels in lipid raft microdomains

Lateral assembly between G-protein-coupled receptors, such as P2Y receptor subtypes, PLC and TRP channels occur most probably at lipid raft/caveolae membrane microdomains. Caveolin-1 specifically binds $G_{\alpha q}$ and stabilizes its activation state resulting in an enhanced Ca^{2+} signal upon activation. More relevant to the present study, it has been demonstrated that hydrophobic interactions between TRP channels and lipid rafts modulate the opening properties of these channels. Therefore, we decided to evaluate ADP-induced $[Ca^{2+}]_i$ responses after acute depletion of cholesterol from the plasma membrane of intact cells with methyl-ß-cyclodextrin (2 mM), which results in perturbation of lipid rafts architecture eventually leading to caveolae disappearance (Thomsen et al., 2002). Micrographs shown in Figure 16G foresee that reduction of membrane cholesterol with methyl-ß-cyclodextrin (200 μM) affects the structural integrity of caveolae in the plasma membrane as suggested by the downregulation of caveolin-1 immunoreactivy in ventricular myofibroblasts (Figure 16H). Pretreatment with methyl-ß-cyclodextrin (2 mM, n=8) depressed ($p<0.05$) the late component of ADP (100 μM)-induced $[Ca^{2+}]_i$ response (phase II), without modifying the initial fast $[Ca^{2+}]_i$ rise (Figure 16G). The effect of methyl-ß-cyclodextrin (2 mM) had a similar pattern to that observed in the presence of LiCl (10 mM), 2-APB (50 μM) and flufenamic acid (100 μM) (see above).

ADP favours cell growth and type I collagen production by ventricular myofibroblasts via $P2Y_1$ receptors activation

Previous reports from the literature demonstrate that ADP promotes fibrosis in a number of different tissues by increasing the proliferation of fibroblasts (Pinheiro et al., 2013b; Garbuzenko et al., 2002), yet the role played by ADP and ADP-preferring P2Y purinoceptors on cardiac fibroblasts from adult rats has not been investigated so far. Incubation of cultured ventricular fibroblasts with ADP or its enzymatically-stable analogue, ADPβS, increased ($p<0.05$) cells proliferation

(MTT assay) from the first week onwards; these compounds were about equipotent when applied at a concentration of 30 μM (Figure 17Ai). Likewise, both ADP and ADPβS (30 μM) enhanced ($p<0.05$) type I collagen production above the control level, but the magnitude of the increments were smaller and shifted towards later time periods when compared to their effects on cells growth (Figure 17Aii).

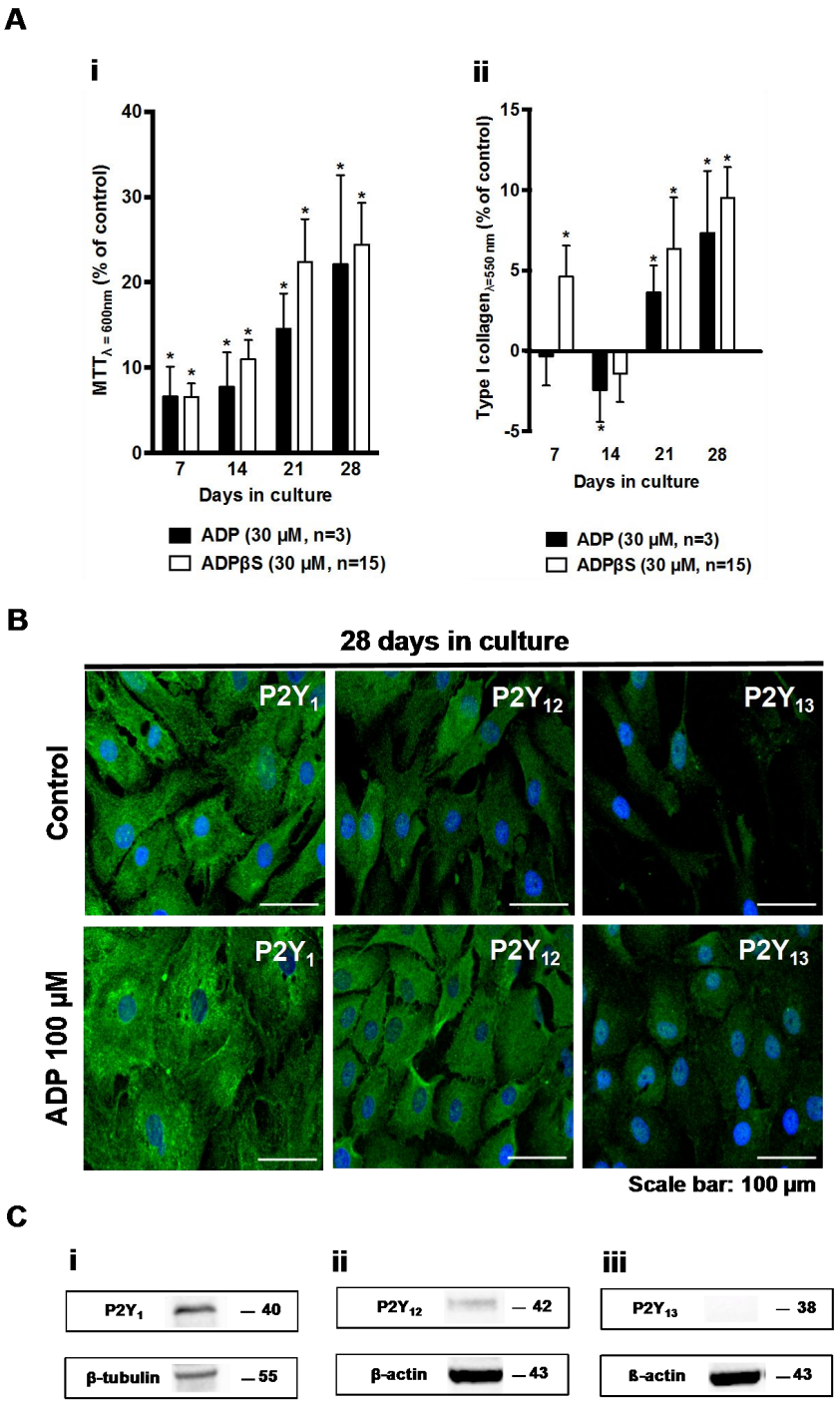

Figure 17 - Role of ADP and its stable analogue ADPβS on cell growth and type I collagen production by fibroblasts from adult rat ventricles grown for 28 days in culture. In panel A the ordinates represent ADP (30 μM)- or ADPβS (30 μM)-induced changes in cell growth (MTT values,

Ai) and type I collagen production (Sirius Red values, Aii) as compared to controls in the absence of the nucleotides at the same points. Zero represents similarity between the two values (nucleotides vs control); positive and negative values represent facilitation or inhibition of either cell growth or type I collagen production relative to control data obtained at the same time points. Each bar represents pooled data from an *n* number of individuals; four replicas were performed for each individual experiment. The vertical bars represent S.E.M. *p<0.05 represent significant differences from control values obtained in the absence of test drugs, Panel B shows $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors immunoreactivity of adult rat ventricular fibroblasts cultured with ADP (100 μM) during 28 days. Confocal micrographs shown are representative of three independent experiments; scale bar is 100 μm. Panel C shows representative immunoblots of $P2Y_1$ (Ci), $P2Y_{12}$ (Cii) and $P2Y_{13}$ (Ciii) receptors obtained from homogenates of ventricular fibroblasts grown in culture for 28 days in control conditions. α-actin and β-tubulin were used as reference proteins. Images are representative of three independent experiments.

Figure 17B shows that after growing cells in culture for 28 days in the presence of ADP (100 μM, n=3), ventricular fibroblasts differentiate into stellate shaped myofibroblasts with protruding dendritic-like processes which still express significant amounts of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors. Immunofluorescence confocal microscopy and Western blot analysis indicate that the non-desensitizing $P2Y_1$ receptor is the most abundant receptor subtype among all ADP-preferring P2Y purinoceptors in ventricular myofibroblasts of adult rats, followed by $P2Y_{12}$ and $P2Y_{13}$ receptor subtypes in that order (Figure 17B and 17C). Cell processes of activated ventricular myofibroblasts exhibit outstanding $P2Y_1$ receptor immunoreactivity compared to $P2Y_{12}$ and $P2Y_{13}$, which receptor staining is sparser. In order to characterize the P2Y receptor subtype involved on ADPβS (30 μM)-induced proliferation and type I collagen production, ventricular fibroblast cell cultures were treated during 28 days with the ADP stable analogue in the presence of selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor antagonists, MRS2179 (0.3 μM, n=6), AR-C66096 (0.1 μM, n=6) and MRS2211 (10 μM, n=6), respectively. Under these experimental conditions, the $P2Y_1$ receptor antagonist, MRS2179 (0.3 μM), was the only compound capable of attenuating ADPβS (30 μM)-induced fibroblast cells growth and type I collagen production (Figure 18), while both AR-C66096 (0.1 μM, n=6) and MRS2211 (10 μM, n=6) were ineffective. On its own, MRS2179 (0.3 μM) marginally decreased ($p<0.05$) type I collagen production by ventricular myofibroblasts at later cultures stages, i.e. days 21 and 28 (Figure 18i and 18iv).

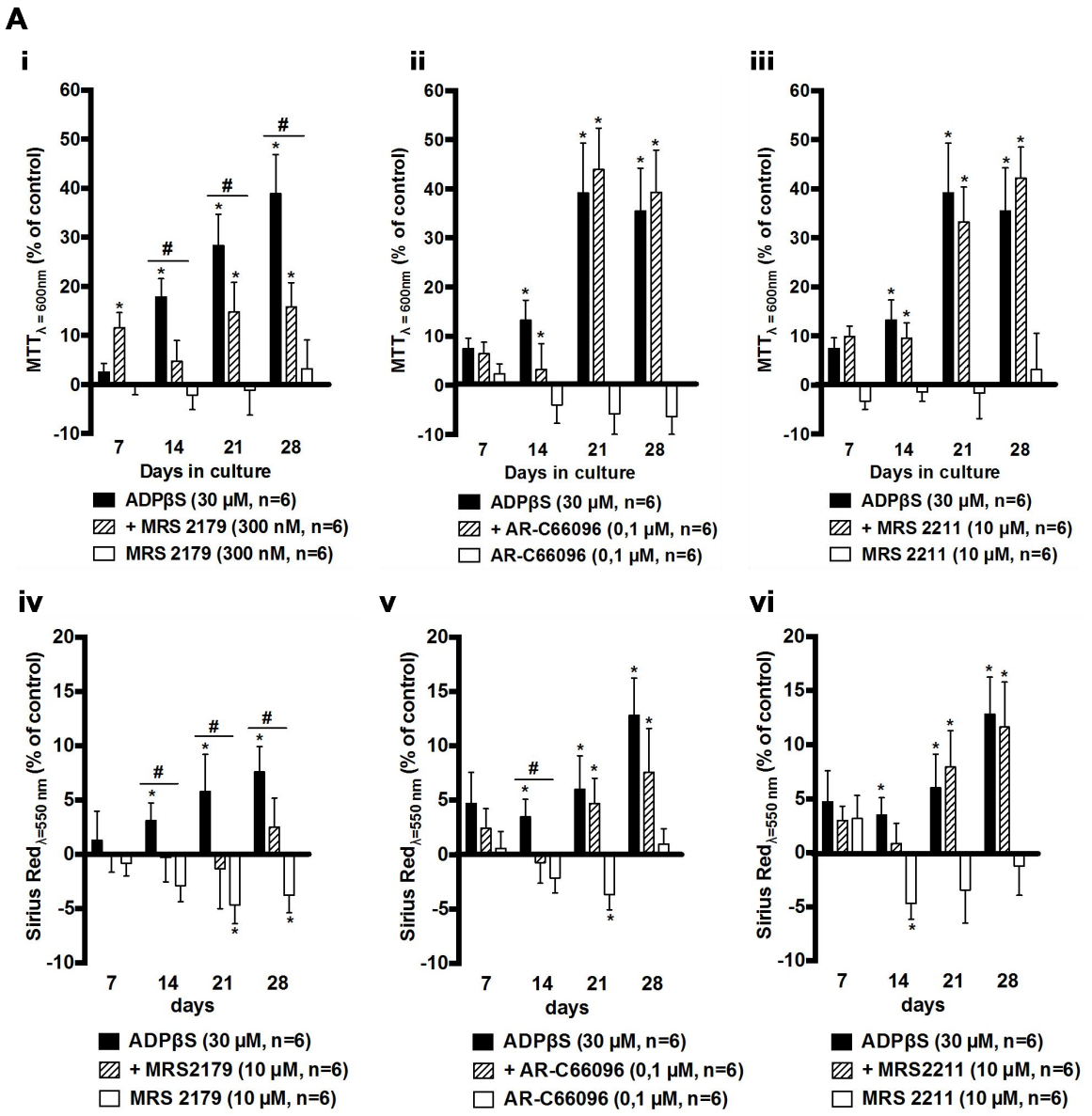

Figure 18 - The pro-fibrotic effect of ADPβS depends on the activation of $P2Y_1$ receptors. ADPβS (30 μM) with or without MRS 2179 (300 nM, a selective $P2Y_1$ antagonist, Ai and Aiv), AR-C 66096 (0.1 μM, a selective $P2Y_{12}$ antagonist, Aii and Av) and MRS 2211 (10 μM, a selective $P2Y_{13}$ antagonist, Aiii and Avi) were incorporated in culture media, and, therefore, contacted with the cells during the whole assay. The ordinates represent changes in cell growth (MTT values, top panels) and type I collagen production (Sirius Red values, bottom panels) compared to controls in the absence of test drugs at the same points. Zero represents similarity between the two values (drugs vs control); positive and negative values represent facilitation or inhibition of cell growth relative to control data obtained at the same time points. Each bar represents pooled data from an *n* number of individuals; four replicas were performed for each individual experiment. The vertical bars represent S.E.M.. *p<0.05 represent significant differences from control values obtained in the absence of test drugs. #p<0.05 represent significant differences from ADPβS (30 μM) alone.

These findings reiterate a long-term pro-fibrotic action of ADPβS on ventricular fibroblasts via dominant activation of the $P2Y_1$ receptor, which differs from the predominance of the $P2Y_4$ receptor on fast $[Ca^{2+}]_i$ transients (see above; Certal et al., 2015). This hypothesis is strengthen by the results presented in

Figure 19, showing that ADPβS (30 μM)-induced fibroblast cells growth was significantly ($p<0.05$) attenuated by U73122 (3 μM, n=6), chelerythrine (3 μM, n=4) and 2-APB (50 μM, n=5), but not SQ22536 (30 μM, n=3), thus confirming the participation of a PLC transduction pathway linked to long-term effects of ADPβS operated by PKC and TRP channels, respectively. .

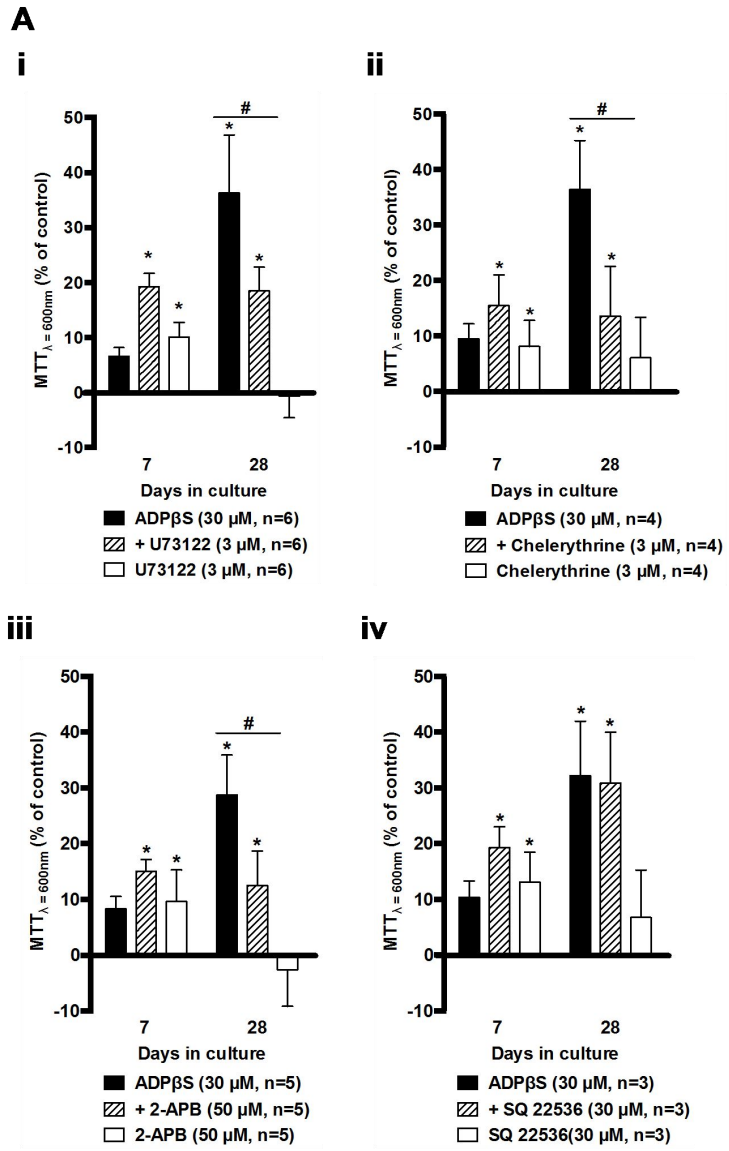

Figure 19 - The proliferative effect of ADPβS on ventricular fibroblasts involves activation of phospholipase C (PLC), protein kinase C (PKC) and TRP channels, but not the adenylate cyclase (AC) pathway. Shown is the variation of cell growth at 7 and 28 culture days caused by continuous application of ADPβS (30 μM) to the culture medium in the absence and in the presence of inhibitors of PLC, PKC, TRP channels, and AC, respectively U73122 (3 μM, Ai), chelerythrine (3 μM, Aii), 2-APB (50 μM, Aiii) and SQ 22536 (30 μM, Aiv). The ordinates represent changes in MTT values compared to control cultures grown in the absence of test drugs at the same time points. Each column represents pooled data from *n* individuals; four replicas were performed for each individual experiment. The vertical bars represent S.E.M.. *$p<0.05$ represent significant differences from control values obtained in the absence of test drugs. #$p<0.05$ represent significant differences from ADPβS (30 μM) alone.

Discussion

In this study, we show that ADP possibly resulting from the extracellular catabolism of released ATP by ectonucleotidases exerts a dual role on $[Ca^{2+}]_i$ transients and proliferation of myofibroblasts from adult rat ventricles through differential activation of fast-desensitizing $P2Y_4$ and long-acting $P2Y_1$ receptors, respectively. Although it has been previously demonstrated that the $P2Y_4$ receptor is the dominant receptor subtype mediating fast $[Ca^{2+}]_i$ transients caused by UTP and ATP in these cells (Certal et al., 2015), we show here that this receptor is also involved in ADP-induced effects. Moreover, our results suggest for the first time that sustained $P2Y_4$ receptor-mediated $[Ca^{2+}]_i$ accumulation produced by ADP might be due to capacitative Ca^{2+} entry through TRP channels opening downstream to $G_{q/11}$-protein-coupled PLC activation in lipid raft microdomains. ADP may also exert long-term pro-fibrotic effects by increasing proliferation and type I collagen production by ventricular myofibroblasts via the activation of ADP-preferring $P2Y_1$ receptors. Unlike fast $[Ca^{2+}]_i$ rises, the proliferative action of ADP may involve phosphorylation of intracellular constituents by PKC subsequently to PLC activation and DAG release from the plasma membrane. The detailed intracellular mechanism(s) underlying the pro-fibrotic effect of ADP via $P2Y_1$ receptors activation remains to be investigated in future studies.

Notwithstanding our results (from immunofluorescence confocal microscopy and Western blot analysis) showing that cultured myofibroblasts from adult rat ventricles express all ADP-preferring receptor subtypes, namely $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$, we failed to demonstrate their activity when measuring $[Ca^{2+}]_i$ oscillations in the presence of subtype selective antagonists, respectively MRS2179, AR-C66096 and MRS2211. Taking into consideration that ADP is competent to activate the rat $P2Y_4$ receptor with a similar potency as ATP and UTP (Bogdanov et al., 1998; Burnstock, 2012), we tested whether this receptor was involved in our experimental conditions. The dominant role of the $P2Y_4$ receptor on ADP-induced $[Ca^{2+}]_i$ transients was inferred by differences in the blocking potency of suramin and reactive blue-2, with the latter being a more effective $P2Y_4$ antagonist than suramin (von Kugelgen, 2006). This assumption is further strengthen by previous findings from our group using the selective $P2Y_4$ receptor agonist, MRS4062, which along with UTP exhibited a similar pharmacology, together with confirmation by confocal microscopy and Western blot analysis that ventricular myofibroblasts

of adult rats express $P2Y_4$ receptors (Certal et al., 2015).

Signals operated by intracellular calcium oscillations are essential for cardiac fibroblasts proliferation, migration and transdifferentiation into (activated) myofibroblasts. Moreover, abnormalities in $[Ca^{2+}]_i$ handling by cardiac cells may determine arrhythmogenesis and heart fibrosis. We report here that myofibroblasts from adult rat ventricles respond to ADP through activation of the PLC transduction pathway downstream $G_{q/11}$-protein-coupled $P2Y_4$ receptors stimulation. The initial $[Ca^{2+}]_i$ rise (phase I) depends on rapid Ca^{2+} mobilization from internal stores that is most likely due to the release of IP_3 from membrane phospholipids. This conclusion is based on the fact that both (1) inhibition of PLC with U73122, and (2) depletion of intracellular Ca^{2+}-stores with thapsigargin plus caffeine, attenuated the fast $[Ca^{2+}]_i$ mobilization by ADP. Adenine nucleoside diphosphates trigger PLC activation followed by the production of IP_3 in neonatal cardiac fibroblasts (Talasila et al., 2009), but as we show here ADP-induced $[Ca^{2+}]_i$ rises are independent on PKC activation by DAG since inhibition of this enzyme with chelerythrine failed to affect the ADP $[Ca^{2+}]_i$ response. The participation of ADP-sensitive $P2Y_{12}$ and $P2Y_{13}$ receptor subtypes negatively coupled to adenylate cyclase (Alexander et al., 2015) was also rejected because selective inhibition of this enzyme with SQ22536 or MDL12,330a did not impact on the magnitude and/or shape of ADP-induced $[Ca^{2+}]_i$ response.

We show here that extracellular Ca^{2+} also plays a role to sustain the late component (phase II) of ADP $[Ca^{2+}]_i$ response. This was assumed because omission of Ca^{2+} (plus EGTA) from the incubation medium significantly attenuated the nucleotide response. Fibroblasts lack voltage-gated Ca^{2+} channels and Ca^{2+} influx from the extracellular milieu is more likely to occur via ion channels activated by PLC downstream of $G_{q/11}$-protein-coupled receptors, such as the $P2Y_4$ subtype. TRP channels are widely expressed proteins forming non-selective cation channels. The vast majority of TRP channels is permeable to Ca^{2+} and directly contributes to cellular Ca^{2+} influx. TRP channel subunits contain six putative transmembrane domains assembled as homo- or heterotetramers. Following PLC activation, breakdown of phosphatidylinositides (PIP_2) leads to the formation of IP_3 and DAG, which may directly activate certain types of canonical TRPC channels (e.g. TRPC2, TRPC3, TRPC6, and TRPC7) in a PKC-independent manner

(Ramsey et al., 2006; Harteneck and Gollasch, 2011). This scenario fits in the results obtained in our study. The presence of TRPC1, TRPC3, TRPC6, and TRPC7 channels has been demonstrated in rat cardiac fibroblasts and they have been implicated in cardiac hypertrophy (Nishida et al., 2007). Other authors also argue that TRPM7, TRPC3, TRPC6 and TRPV4 are crucial for fibroblast-to-myofibroblast transition and cardiac arrhythmogenesis (Adapala et al., 2013; Davis et al., 2012; Du et al., 2010; Tadevosyan et al., 2012; reviewed in Thodeti et al., 2013). Attenuation of spontaneous $[Ca^{2+}]_i$ oscillations by PLC and TRP channel inhibitors has been observed in cultured human cardiac fibroblasts, but information is still lacking regarding the mechanisms how Ca^{2+} signals regulate the biological activity of these cells and cardiac remodeling under pathological conditions. Unfortunately, the pharmacology of most TRP channels is poorly developed thus preventing the investigation of more definitive involvement of these channels on Ca^{2+} signaling by ventricular myofibroblasts. Definitive characterization of TRP channel subtypes is further aggravated because mixed activation properties often occur as a consequence of heteromerization of several pore forming subunits and/or of tight association with diverse membrane proteins to form signaling complexes (e.g. NCX, CaM, PLC, IP_3 receptor). Notwithstanding this, we used two broad spectrum TRP inhibitors, 2-APB and flufenamic acid, which have been instrumental to explore the participation of these channels in diverse experimental settings (Ramsey et al., 2006; Harteneck and Gollasch, 2011; Guinamard et al., 2013). Our findings indicate that non-selective blockade of TRP channels significantly decreased ADP-induced capacitative Ca^{2+} entry resulting in attenuation of the $[Ca^{2+}]_i$ plateau (phase II) without changing the initial $[Ca^{2+}]_i$ rise (phase I). Response profile to ADP in the presence of TRP channels inhibition by 2-APB and flufenamic acid resembled that obtained in the absence of external Ca^{2+} (plus EGTA) and in the presence of LiCl, which inhibits inositol monophosphatase thus preventing the synthesis of phosphoinositides (Hanson, 1991) and the regeneration of adequate PIP_2 levels to be hydrolysed to IP_3 and DAG (Berridge and Irvine, 1989).

Another possibility to increase $[Ca^{2+}]_i$ entry into ventricular myofibroblasts linked to ADP-sensitive $P2Y_4$ receptor activation is a putative interplay between this receptor and the Na^+/Ca^{2+}-exchanger (NCX) operating in the reverse mode. It has been demonstrated that NCX may contribute to intracellular Ca^{2+} overload in

cardiomyocytes during myocardial infarction (Chen and Li, 2012). Even though this situation may putatively contribute to increase the extracellular levels of adenine and uracil nucleotides (Wihlborg et al., 2006), there are no reports available linking NCX and P2Y purinoceptors activity in cardiac fibroblasts. Under the present experimental conditions, inhibition of NCX with KB-R7943 (50 μM) did not affect $[Ca^{2+}]_i$ signals induced by ADP in ventricular myofibroblasts.

Regulation of lateral association between G-protein-coupled receptors, PLC activation and opening of TRP channels by lipid rafts has been documented in several cell types. Here we show that lipid rafts disruption by cholesterol depletion with methyl-ß-cyclodextrin (Thomsen et al., 2002) significantly attenuated the plateau of ADP-induced $[Ca^{2+}]_i$ response (phase II) without affecting the fast $[Ca^{2+}]_i$ rise (phase I). Taken together, these results suggest that hydrophobic interactions between $P2Y_4$ and PLC-linked TRP channels activation leading to capacitative Ca^{2+} entry in ventricular myofibroblasts occur most likely in lipid raft microdomains, whereas the initial $[Ca^{2+}]_i$ rise depends on IP_3 release from the plasma membrane that is required for rapid Ca^{2+} mobilization from internal reservoirs. Using immunofluorescence confocal microscopy we also showed that depletion of cholesterol from lipid rafts by methyl-ß-cyclodextrin may disrupt the architecture of caveolae (Schönfelder et al., 2006) and decrease the expression of caveolin-1 (Williams and Lisanti, 2004) in these cells. Unlike other types of G protein subunits, $G_{\alpha q}$ binds to the scaffold domain of caveolin-1 and -3, and this binding is strengthened when $G_{\alpha q}$ is activated (Sengupta et al., 2008). The association site between caveolin and $G_{\alpha q}$ differs from $G_{\alpha q}$ and PLC, and so $G_{\alpha q}$ continues to signal through PLC when bound to caveolae. The interaction between caveolin proteins and $G_{\alpha q}$ has the effect of targeting $G_{\alpha q}$ and its associated receptors to caveolae domains (Calizo and Scarlata, 2012). Since the interactions between caveolin-1 and $G_{\alpha q}$ are strengthened upon activation, localization of $G_{\alpha q}$ to caveolae can stabilize its activated state and enhance Ca^{2+} signals. Taking this into account and considering that diverse phenotypes induced by altered caveolar gene function encoding mostly caveolin-1 are involved in cardiomyopathy, cardiac hypertrophy, cardiac arrhythmias and exercise intolerance (reviewed in Cheng and Nichols, 2015), we may speculate about the pharmacological manipulation of rapid coupling/uncoupling of P2Y purinoceptors and signal transduction pathways at

lipid rafts/caveolae microdomains as an important clue to design drugs for therapeutic intervention in cardiac remodeling.

Proliferation and type I collagen production are key events in the fibrotic response to cardiac remodelling. It is known that extracellular nucleotides are able to modulate these processes in various cell types (Chen et al., 2012; Weber, 2000). Combining the findings from previous studies showing that UTP (Braun et al., 2010; Certal et al., 2015) and ATP (Chen et al., 2012) are pro-fibrotic in cardiac fibroblasts, together with the fact that ATP may be rapidly converted extracellularly into ADP by membrane-bound ectonucleotidases, this prompted for the interrogation about the contribution of ADP to nucleotides-induced cell proliferation and type I collagen production by myofibroblasts from adult rat ventricles. Our data demonstrate that these cells respond equally to ADP and to its stable analogue, ADPβS, by increasing cells growth and type I collagen production through the activation the $P2Y_1$ receptor, the most abundant ADP-preferring receptor subtype in ventricular myofibroblasts. This was suggested because the selective $P2Y_1$ antagonist, MRS2179, but not AR-C66096 and MRS2211, significantly attenuated myofibroblast cells proliferation (MTT assay) and type I collagen production (Sirius Red staining assay). Thus, in spite of the presence of ADP-sensitive $P2Y_1$ and $P2Y_{13}$ receptors in cardiac myofibroblasts from neonatal rats (Talasila et al., 2009), the pathophysiological role of the $P2Y_{13}$ receptor in adult animals requires further elucidation. On its own, the $P2Y_1$ receptor antagonist, MRS2179, decreased type I collagen production at culture days 21 and 28, without much affecting the proliferation of the cells. These results suggest that tonic activation of the $P2Y_1$ receptor may exert a preferential effect on extracellular matrix deposition than on cells growth. The pro-fibrotic effect of ADP is consistent with other studies demonstrating that the nucleotide can modulate cellular proliferation in various cell types, like subcutaneous connective tissue fibroblasts, keratinocytes, and lung fibroblasts (Pinheiro et al., 2013b; Gendaszewska-Darmach and Kucharska, 2011), although one study argued that stimulation of the $P2Y_1$ receptor may inhibit noradrenaline-induced DNA synthesis in neonatal cardiac fibroblasts (Zheng et al., 1998).

The lack of an ADP-induced $P2Y_4$ receptor-mediated effect on cells growth and type I collagen production is not surprising because this receptor desensitizes rapidly in cardiac fibroblasts by repeated applications or prolonged contact with its

agonists (see Certal et al., 2015). It has been demonstrated that P2 purinoceptors association to lipid rafts/caveolae may be regulated by agonists; indeed, rapid internalization and slow cell surface recycling of the receptors have been associated with prolonged fading of the responses in the presence of agonists (D'Ambrosi and Volonté, 2013). In contrast to the $P2Y_4$ receptor subtype, we confirmed by immunofluorescence confocal microscopy that the density of the $P2Y_1$ receptor remained steady after growing the cells in culture during 28 days in the presence of 100 μM ADP. If something has to be noted in these experiments is the transition of fibroblasts to more mature/activated myofibroblasts exhibiting a typical stellate-shaped morphology, which is usually observed in situ when these cells are committed to myocardial remodelling (Rohr, 2011).

While PLC-coupling to Ca^{2+}-permeable TRPC3 and TRPM7 channels has been shown to be up-regulated in human atrial fibroblasts (Du et al., 2010; Harada et al., 2012), the TRPC6 channel (that is also sensitive to blockade by 2-APB) along with the mechanically-stimulated TRPV4 channel may be critical for ventricular fibroblast differentiation (Thodeti et al., 2013). The signalling cascades by which TRP channels regulate fibroblast differentiation to myofibroblasts during cardiac remodelling may differ, but all culminate in increased (-SMA expression that is the primary contractile protein in myofibroblasts. It is interesting to note that transgenic expression of human TRPC3 channel in mouse heart is associated with increased capacitative Ca^{2+} entry, activation of Erk1/2 and/or calcineurin-nuclear factor of activated T cells (NFAT) in vivo, cardiomyopathy, and increased hypertrophy after neuroendocrine agonist or pressure overload stimulation (Nakayama et al., 2006). Genetic ablation of TRPC6 gene in fibroblasts made them refractory to inducers of myofibroblast transformation (e.g. TFGβ, angiotensin-II) and TRPC6 null mice had greater death rates after myocardial infarction due to defective scar formation with wall rupture; conversely, TRPC6 induction led to enhanced Ca^{2+} entry in response to agonist stimulation suggesting that Ca^{2+} is likely an effector of myofibroblast transformation (Davis et al., 2012). Moreover, a synergistic interplay between $P2Y_1$-mediated responses and TRPV4 channel activation involving the PKC pathway has recently been proposed in satellite glial cells of sensory ganglia (Rajasekhar et al., 2015). Aligned with these findings, we show here for the first time that the pro-fibrotic effect of ADP involves

$P2Y_1$ receptors coupled to U73122-sensitive PLC pathway, which results in enduring activation of PKC and TRP channels blocked by chelerythrine and 2-APB, respectively. It remains, however, to be elucidated the TRP channel subtype most involved in long-term effects of ADP on myofibroblasts from adult rat ventricles.

The time-course and magnitude of the proliferative effect of ADP on ventricular myofibroblasts (via $P2Y_1$ receptors activation) was very similar to that observed upon activating $P2Y_2$ receptors with UTP in the same cell type, but ADP and its stable analogue, ADPβS, were more potent than UTP regarding type I collagen production and, hence, extracellular matrix deposition (see Certal et al., 2015). The power ratio between these two pro-fibrotic receptors and the anti-proliferative effect recently attributed to the $P2Y_{11}$ receptor (Certal et al., 2015) is still unknown and deserves to be fully explored in the future. Despite this uncertainty, inhibition of adenylate cyclase activity with SQ22536 did not affect cells growth induced by ADPβS. Results indicate that, in contrast to ATP and UTP, the pro-fibrotic effect of adenine nucleoside diphosphates is apparently unrestrained by synchronous activation of the anti-fibrotic $P2Y_{11}$ receptor coupled to the adenylate cyclase/cyclic AMP/EPAC (exchange protein directly activated by cyclic AMP) pathway.

Conclusions

In summary, adenine and uracil nucleotides can be released from endothelial cells, cardiomyocytes and fibroblasts in the heart during stressful pathological conditions (e.g. myocardial infarction, vascular stress forces, hypoxia, pressure overload). Besides the most studied mechanisms underlying cardiomyocyte hypertrophic responses, nucleotides may promote fibroblast growth and fibroblast-to-myofibroblast transition leading to cardiac fibrosis. Repercussions of myocardial fibrosis are decrease in compliance, arrhythmias, diastolic dysfunction and accompanying heart failure. Despite its pathophysiological relevance, little information is available on the fate and activity of released nucleotides including binding and autocrine/paracrine activation of P2 purinoceptors in cardiac fibroblasts. ATP and UTP are strongly pro-fibrotic through the activation of $P2Y_2$ receptors, which are highly expressed in ventricular fibroblasts (reviewed in Lu and Insel, 2014). Besides the $P2Y_2$ receptor, ATP may

directly activate ionotropic P2X4 and P2X7 receptors on cardiac fibroblasts to stimulate cell proliferation. ATP-mediated pro-fibrotic effects may be counteracted by indirect stimulation of G_s-protein-coupled A_{2B} receptors by adenosine generated by ATP hydrolysis via ectonucleotidases (reviewed in Ferrari et al., 2015). More recently, it has been proven that manoeuvres that increase intracellular cyclic AMP diminish proliferation and type I collagen production by cardiac fibroblasts; for instance, the previous unrecognized anti-fibrotic role of UTP-sensitive $P2Y_{11}$ receptors implicates such a mechanism in ventricular fibroblasts (Certal et al., 2015). Here, we added valuable information regarding the unrestrained pro-fibrotic action of ADP, a by-product of the extracellular hydrolysis of ATP by ectonucleotidases. Data show that ADP strengthens the effect of released ATP and UTP on $[Ca^{2+}]_i$ accumulation through activation of fast desensitizing $P2Y_4$ receptors, while it promotes myofibroblast cells growth and type I collagen production via long-acting $P2Y_1$ receptors. The mechanisms underlying ADP effects involve PLC activation linked (1) to the release of IP_3 from the plasma membrane and its diffusion to the endoplasmic reticulum leading to fast Ca^{2+} recruitment from internal stores, and subsequent (2) capacitative Ca^{2+} entry via opening of TRP channels compartmentalized in lipid rafts microdomains. Enrolment of a PKC-mediated pathway was verified only by looking to the long-term proliferative effect of ADP. Thus, targeting the mechanisms controlling purinergic signals revealed in this study may be of therapeutic interest to design new drug strategies to prevent cardiac fibrosis and its deleterious effects on myocardial function.

CHAPTER 4. DISCUSSION AND CONCLUSIONS

Cardiac fibroblasts play a crucial role in cardiac remodeling, electrophysiology and hemodynamic alterations following myocardial infarction (Porter and Turner, 2009). As mentioned early, ATP and UTP are released in huge amounts during ischemia and reperfusion and may act as autocrine and/or paracrine mediators via the activation of a variety of P2 purinoceptors (Wihlborg et al., 2006). In order to understand the underlying purinergic signaling mechanisms that could be involved in the regulation of fibroblast cells function and cell-to-cell communication in the heart, we decided to isolate its major cell constituent, cardiac fibroblasts. In this thesis, we evaluated the role of metabotropic P2Y receptors through their effect on $[Ca^{2+}]_i$ oscillations, given that Ca^{2+} is an intracellular messenger that controls a huge range of cellular processes. In addition, we also selected the assessment of proliferation and type I collagen synthesis by cardiac fibroblast cell cultures because of the core importance of these events to the mycardium remodeling process.

Our results provided evidences that cardiac fibroblasts really undergo a phenotype switch to myofibroblasts *in vitro* given that, under our experimental conditions, cardiac fibroblasts exhibited positive immunoreactivity for α-SMA, in addition to specific markers of these cells, vimentin and DDR2. As a matter of fact, the differentiation of cardiac fibroblasts into a myofibroblast phenotype was described in two-dimension cultures in the absence of additional stimulation (Porter et al., 2004; Jarvis et al., 2006). Thus, the cells used in this work resemble those undergoing myofibroblastic differentiation *in vivo* secondary to pathological conditions and/or to mechanical, inflammatory, chemical stimulation.

Since adenine and uracil nucleotides play important roles in the propagation/amplification of cells communication (Burnstock, 2006; Gendaszewska-Darmach and Kucharska, 2011), we tried to elucidate the repercussions of these nucleotides on cardiac fibroblasts activation. Our findings demonstrate that ATP, ADP, UTP and UDP increased $[Ca^{2+}]_i$ accumulation. In this regard, nucleoside triphosphates, like ATP and UTP, were more potent than their diphosphate metabolites, ADP and UDP, respectively. In view of this, one may conclude that the extracellular catabolism of released nucleoside triphosphates by ectonucleotidases may establish a set point on their effects on $[Ca^{2+}]_i$ signaling in cardiac fibroblasts.

This study contributes to functionally characterize the role of UTP- and ADP-senstive P2Y receptors in cardiac fibroblasts, given that there was a gap in our knowledge regarding this issue in the literature. UTP exerts its effects acting directly through $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors, which are the only P2 receptor subtypes sensitive to both ATP and UTP (Talasila et al., 2009). The presence of these receptors in fibroblasts from adult rat ventricles was demonstrated here by immunofluorescence confocal microscopy and Western blot analysis. Data indicate that UTP exerts a dual role via the activation of fast-desensitizing $P2Y_4$ and long-acting $P2Y_2$ receptors, which are responsible for $[Ca^{2+}]_i$ transients and growth of ventricular fibroblasts, respectively. UTP may also exert its effects via the $P2Y_6$ receptor after its extracellular conversion to UDP through NTPDases action (Burnstock, 2007). Using a selective agonist and antagonist of the $P2Y_6$ receptor we failed to detect any functional role for this receptor on cardiac fibroblasts.

We reveal here for the first time that synchronous activation of the $P2Y_{11}$ receptor by released ATP counteracts the pro-fibrotic effect of the $P2Y_2$ receptor stimulation by UTP. Moreover, the prominent role of the $P2Y_{11}$ receptor in controlling cardiomyocyte contractility may point towards this receptor being an important target for therapeutic intervention to prevent deleterious cardiac remodeling under various pathological conditions. We also show here that the $P2Y_{11}$ receptor is involved in sustaining high intracellular Ca^{2+} levels during the second phase of UTP-induce response. The mechanism underlying the synergism between $P2Y_4$ (dominant receptor) and $P2Y_{11}$ purinoceptors regarding UTP-induced $[Ca^{2+}]_i$ accumulation may depend on endogenous ATP outflow from stimulated cardiac fibroblasts via Cx43-containing hemichannels, which can be blocked by carbenoxolone and H1152 (an inhibitor of Rho kinase-operated Cx43-pore permeability).

Released ATP can be rapidly hydrolyzed extracellularly to ADP by membrane-bound ectonucleotidases, but until now no functional characterization of the putative roles of this intermediate metabolite has been attempted on cardiac fibroblast cells function and myocardial remodeling. Using immunofluorescence confocal microscopy and Western blot analysis, we confirmed the presence of ADP-sensitive $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor subtypes in cardiac fibroblasts of adult rat ventricles. Notwithstanding this, the most abundant $P2Y_1$ receptor

subtype was the only that appears to have a role in adult rat ventricular fibroblasts by increasing cells proliferation and type I collagen production. The magnitude of the $P2Y_1$ receptor-mediated proliferative effect on ventricular myofibroblasts was very similar to that observed upon activating $P2Y_2$ receptors with UTP, but ADP and its stable analogue ADPβS were more potent than UTP on type I collagen production by these cells. Whether this discrepancy is due to the anti-fibrotic effect of UTP operated by synchronous activation of G_s-protein-coupled $P2Y_{11}$ receptor remains to be investigated. As a matter of fact, inhibition of adenylate cyclase activity (with SQ22536) did not modify cells proliferation and type I collagen production induced by ADPβS, as it did when UTP was used instead. Data, thus, suggest that ADP-mediated effects on fibroblast cells proliferation and matrix deposition is apparently unrestrained by synchronous activation of the anti-fibrotic $P2Y_{11}$ receptor coupled to the adenylate cyclase/cyclic AMP/EPAC pathway, in contrast to that observed with UTP.

Much to our surprise, none of the ADP-sensitive P2Y receptors ($P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$) seem to be involved in acute $[Ca^{2+}]_i$ transients in ventricular fibroblasts of adult rats. ADP concentration-dependenly increased $[Ca^{2+}]_i$ accumulation through a non-canonical activation of fast desensitizing $P2Y_4$ receptors (Bogdanov et al., 1998; Burnstock, 2012), although with a lesser potency than that observed for UTP and ATP under the same experimental conditions. The mechanisms underlying $[Ca^{2+}]_i$ transients induced by $P2Y_4$ receptors activation implicate PLC stimulation and the release of IP_3 and DAG from membrane phospholipids (PIP_2). Whereas soluble IP_3 may trigger the $P2Y_4$-induced fast $[Ca^{2+}]_i$ rise by favoring the recruitment of Ca^{2+} from thapsigargin-sensitive internal stores, DAG might promote the opening of 2-APB-sensitive TRP channels leading to capacitative Ca^{2+} influx from the extracellular milieu to sustain the plateau phase of the $[Ca^{2+}]_i$ response. Lateral assembly of the $P2Y_4$ receptor, $G_{q/11}$ protein, PLC and TRP channels may occur at lipid rafts/cavelae microdomains, because plasma membrane depletion of PIP_2 and cholesterol with LiCl and methyl-β-cyclodextrin, respectively, equally downsized the late $[Ca^{2+}]_i$ plateau. It is known that $G_{q/11}$ binding to caveolin-1 is strengthen by ($P2Y_4$) receptors activation, which contributes to stabilization of PLC within caveolae resulting in increased Ca^{2+} signals (Calizo and Scarlata, 2012). Interestingly,

activation of $G_{q/11}$ protein-linked mechanisms also favour activation of adenylate cyclase by G_s-protein-coupled receptors, which may additionally explain the synergism between $G_{q/11}$-coupled $P2Y_4$ and G_s-linked $P2Y_{11}$ receptors on $[Ca^{2+}]_i$ transients when mutual ligands were used (e.g. UTP or ATP), but not when only the $P2Y_4$ receptor was activated by ADP.

Ca^{2+} permeable TRP channels are widely expressed in many cell types, including cardiac fibroblasts. Four different TRP channels, TRPM7, TRPC3, TRPC6 and TRPV4, were found to be critical for fibroblasts differentiation to myofibroblasts (Adapala et al., 2013; Davis et al., 2012; Du et al., 2010). Other groups also claimed the presence of TRPC1, TRPC3, TRPC6, and TRPC7 in rat cardiac fibroblasts (Nishida et al., 2007). More importantly, all these channels have been involved in fibrotic heart disease opening the doors for the development of drugs targeting TRP channels modulation. The poor pharmacology of most TRP channels prevented a more definitive functional characterization of the subtypes involved in nucleotide-induced $[Ca^{2+}]_i$ transients and growth of ventricular fibroblasts.

Association between transmembrane receptors and signaling molecules within lipid rafts/caveolae provides an enriched environment for protein-protein interactions necessary for signal transduction. Receptors may translocate into submembrane compartments after ligand binding curtailing there activity, but others can move in the opposite direction favoring their actions (D'Ambrosi et al., 2013). Disruption of cholesterol from submembrane compartments can shift the purinergic receptors from lipid raft/caveolar to non-raft/non-caveolar fraction, and thereby abolish their ability to activate phospholipid-dependent signaling pathways and to integrate additional lipid-controled signaling events. In this context, it is tempting to speculate about the possibility to target rapid coupling/uncoupling subtype-specific purinergic receptors from signal transduction pathways by changing lipid rafts/caveolae microenvironment as an important clue for designing drugs for therapeutic intervention in cardiac remodeling.

In summary, data presented here show that UTP-sensitive $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors differentially affect $[Ca^{2+}]_i$ transients and proliferation of rat ventricular myofibroblasts. Activation of the $G_{1q/11}$-coupled $P2Y_4$ receptor is the main responsible for UTP-induced $[Ca^{2+}]_i$ transients, which may result in ATP release to the extracellular fluid and subsequent activation of $P2Y_{11}$ receptors

linked to G_s proteins and adenylate cyclase/cyclicAMP/EPAC pathway. Besides the expression of ADP-preferring $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors has been detected in fibroblasts isolated from adult rat ventricles, ADP-induced $[Ca^{2+}]_i$ transients are more likely mediated through the activation of $P2Y_4$ receptors coupled to the phospholipase C / IP_3 pathway. Inhibition of TRP channels, as well as disruption of lipid rafts microenvironment and PIP_2 turnover, all affected the plateau phase of ADP-induced $[Ca^{2+}]_i$ response. More importantly, our results show that long-term activation of $P2Y_2$ and/or $P2Y_1$ receptors by UTP and/or ADP, respectively, increase fibroblast cells proliferation. Our findings also demonstrate for the first time that the pro-fibrotic effects of nucleoside triphosphates (UTP and ATP), but not ADP, may be counteracted by the activation of $P2Y_{11}$ receptors. Thus, we propose that the anti-fibrotic effect of $P2Y_{11}$ receptors might be used as a novel therapeutic target to prevent cardiac fibrosis.

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